IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Stephen Alan Jobling *et al.*	Confirmation No.:	3594
Serial No.:	10/632,341	Group Art Unit:	1638
Filed:	August 1, 2003	Examiner:	Brent T. Page
FOR:	PLANTS AND PLANT PRODUCTS		

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

Table of Contents

(1)   Real Party in Interest

The real parties in interest are: (a) Brunob II BV, Velperweg 76, 6824 BM Arnhem, the
Netherlands, holding legal title to the application; and (b) National Starch LLC, 10 Finderne
Avenue, Bridgewater, NJ 08807, holding the beneficial title to the application.

(2)   Related Appeals and Interferences

There are no pending related appeals or interferences.

(3)   Status of Claims

Claims 1-35, 44, and 53 are cancelled.

Claims 36-43 and 45-52 are rejected.

A Notice of Appeal was filed on January 8, 2008.

(4)   Status of Amendments

Amendments to claims 36-42 and 44-53, filed in response to the final Office Action, have been entered by the Examiner, according to the Advisory Action mailed on February 8, 2008. Claims 44 and 53 are being cancelled herewith. No amendments are being submitted herewith.

(5)   **Summary of Claimed Subject Matter**

The present claims are directed to methods of producing starches, starches produced by the claimed methods, and plants.

Independent claim 36 is drawn to a method of producing starch by stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is extracted from the plant. The starch has a viscosity onset temperature reduced by at least about 12°C when compared to starch extracted from equivalent, modified plants. Support for claim 36 can be found in the specification as filed, *e.g.*, at page 10, first full paragraph (lines 6-11); and at page 14, last paragraph, lines 27-31 and carrying over to page 15, line 1.

Independent claim 37 is drawn to a method of producing starch by stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is extracted from the plant. The starch has an endotherm onset temperature reduced by at least about 15°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 37 can be found in the specification as filed, *e.g.*, at page 15, first full paragraph, lines 6-11; and the results presented at page 20, last paragraph, lines 25-26, carrying over to page 21, lines 1-3 and Table 3.

Independent claim 38 is drawn to a method of producing starch by stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The

first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second

nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is

extracted from the plant. The starch has an endotherm onset temperature reduced by at least

about 17°C when compared to starch extracted from equivalent, unmodified plants. Support for

claim 38 can be found in the specification as filed, *e.g.*, at page 15, first full paragraph, lines 6-

11; and the results presented at page 20, last paragraph, lines 25-26, carrying over to page 21,

lines 1-3 and Table 3.

Independent claim 39 is drawn to a method of producing starch by stably transforming a

potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The

first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second

nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is

extracted from the plant. The starch has an increased amount of starch molecules with a degree

of polymerization of 6 to 12, when compared to starch extracted from equivalent, unmodified

plants. Support for claim 39 can be found in the specification as filed, *e.g.*, at page 16, first

paragraph, lines 1-9, especially lines 8-9.

Independent claim 40 is drawn to a method of producing starch by stably transforming a

potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The

first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second

nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is

extracted from the plant. The starch has a decreased amount of starch molecules with a degree of

polymerization of 15 to 24, when compared to starch extracted from equivalent, unmodified

plants. Support for claim 40 can be found in the specification as filed, *e.g.*, at page 16, first

paragraph, lines 1-9, especially lines 8-9.

Independent claim 41 is drawn to a method of producing starch by stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is extracted from the plant. The starch has about a two fold increase in starch molecules with a degree of polymerization of 6 to 7 and a depletion of starch molecules with a degree of polymerization between 15 and 20, when compared to starch extracted from equivalent, unmodified plants. Support for claim 41 can be found in the specification as filed, *e.g.*, at page 16, first paragraph, lines 1-9, especially lines 8-9.

Independent claim 42 is drawn to a method of producing starch by stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. The starch is extracted from the plant. The starch has an endotherm onset temperature of less than about 50°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 42 can be found in the specification as filed, *e.g.*, at page 21, in Table 3.

Independent claim 45 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has a viscosity onset temperature reduced by at least about 12°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 45 can be found in the specification as filed, *e.g.*, at page 10, first full

paragraph, lines 6-11; and at page 14, last paragraph, lines 27-31 and carrying over to page 15, line 1.

Independent claim 46 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has an endotherm onset temperature reduced by at least about 15°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 46 can be found in the specification as filed, *e.g.*, at page 15, first full paragraph, lines 6-11; and the results presented at page 20, last paragraph, lines 25-26, carrying over to page 21, lines 1-3 and Table 3.

Independent claim 47 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has an endotherm onset temperature reduced by at least about 17°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 47 can be found in the specification as filed, *e.g.*, at page 15, first full paragraph, especially lines 6-11; and the results presented at page 20, last paragraph, lines 25-26, carrying over to page 21, lines 1-3 and Table 3.

Independent claim 48 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has an increased amount of starch molecules with a degree of polymerization of 6 to 12 when compared to starch extracted from

equivalent, unmodified plants. Support for claim 48 can be found in the specification as filed, *e.g.*, at page 16, first full paragraph, lines 1-9, especially lines 8-9.

Independent claim 49 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has a decreased amount of starch molecules with a degree of polymerization of 15 to 24 when compared to starch extracted from equivalent, unmodified plants. Support for claim 49 can be found in the specification as filed, *e.g.*, at page 16, first paragraph, lines 1-9, especially lines 8-9.

Independent claim 50 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has about a two fold increase in starch molecules with a degree of polymerization of 6 to 7 and a depletion of starch molecules with a degree of polymerization between 15 and 20 when compared to starch extracted from equivalent, unmodified plants. Support for claim 50 can be found in the specification as filed, *e.g.*, at page 16, first paragraph, lines 1-9, especially lines 8-9.

Independent claim 51 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has an endotherm onset temperature of less than about 50°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 51 can be found in the specification as filed, *e.g.*, at page 21, in Table 3.

Independent claim 52 is drawn to a plant that includes at least a first and a second heterologous antisense nucleic acid sequence. The first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme. Starch extracted from the plant has an endotherm onset temperature of less than about 44°C when compared to starch extracted from equivalent, unmodified plants. Support for claim 52 can be found in the specification as filed, *e.g.*, at page 15, first full paragraph, lines 6-11, and at page 21, in Table 3.

(6) Grounds of Rejection to Be Reviewed on Appeal

Whether claims 36-43 and 45-52 are unpatentable under 35 U.S.C. § 112, first paragraph as being based on a nonenabling disclosure.

Whether claims 36-43 and 45-52 are unpatentable under 35 U.S.C. § 112, first paragraph as lacking written description.

(7)   **Argument**

Claims 36-43 and 45-52 were rejected under 35 U.S.C. § 112, first paragraph as

allegedly lacking written description and enablement (Advisory Action mailed on February 8,

2008, Continuation Sheet page 2). These rejections are respectfully traversed.

I. Enablement Rejections under 35 U.S.C. § 112, First Paragraph

The Applicants note with appreciation that the Office has entered claim amendments

presented in the Response to the Final Office Action filed on January 8, 2008. The Office,

however, has maintained the enablement rejections for the amended claims. According to the

Advisory Action, at page 2:

> [t]he claim amendments do not address or overcome the 112 1st paragraph
> rejections as they apply to multitudes of sequences that may be considered as
> either a potato starch synthase II or a potato starch synthase III gene when absent
> a SEQ ID NO:. Applicant's *[sic]* urge that one of skill in the art would appreciate
> and understand which sequences constitute a potato starch synthase II or a potato
> starch synthase III gene. This is not persuasive because Applicants do not teach
> the features of the SEQ ID NOs that are required for starch synthase II or starch
> synthase III function. Due to the nature of the invention requiring a particular
> starch content, the activity of the starch synthases used and therefore the exaxt
> *[sic]* SEQ ID NO: used is crucial to obtaining the starch as claimed by the instant
> application.

Applicants disagree and submit that the specification fully enables skilled practitioners *to*

make and *use* the claimed methods and plants.

A.   The Specification Enables Skilled Practitioners to Make

and Use the Claimed Embodiments

According to the Manual of Patent Examining Procedure (MPEP), the enablement

analysis of an application disclosure "requires a determination of whether that disclosure,

when, filed, contained sufficient information regarding the subject matter of the claims as

to enable one skilled in the pertinent art *to make and use* the claimed invention." MPEP

§ 2164.01 (emphasis added).

Here, the specification gives ample examples of *how to make* the claimed plants,

and how to carry out the claimed methods. For example, at pages 16-22, Example 1

describes in depth one embodiment of transforming potato plants with antisense potato

starch synthase II (SSII) and synthase III (SSIII), followed by extracting and analyzing

starch from the transformed plants. Example 1 provides Accession Numbers for the

sequences that were used in the experiments (see, *e.g.*, page 17, sections a) and b)), and

those skilled in the art would similarly be able to find sequences and references relevant

to potato SSII and SSIII in public databases.

Further, the present specification enables those skilled in the art *to use* the claimed

plants, and methods. For example, according to the specification at page 4, second full

paragraph, during use, starches obtainable by the claimed methods and from the claimed

plants (*e.g.*, starches having a reduced viscosity onset temperature) could be processed

with reduced energy and under milder processing conditions. Moreover, use of potato

starches as food products was well known in the art at the time of filing the application

(see, *e.g.*, "Field of the Invention" of the specification at page 1). Thus, skilled artisans

could use the starches of the present invention to formulate a food product without undue

experimentation.

B.   No Undue Experimentation Is Necessary to Carry out the Claimed Embodiments

Further, no undue experimentation would be required in carrying out the claimed methods and obtaining the claimed plants. At the time of filing the application, skilled practitioners were able to find potato SSII and SSIII sequences in public databases. For example, a search in the National Center for Biotechnology Information (NCBI) nucleotide database for potato starch synthase II brings up 5 records for "core nucleotides," *i.e.*, non-EST (Expressed Sequence Tag) and non-GSS (Genome Sequence Survey). Of the five core nucleotide records, three are for *Arabidopsis thaliana* (thale cress) and *two* are for *Solanum tuberosum* (potato). The two potato records are: (1) *X87988* (the sequence referenced *in the present application at page 17*, although with reversed numbers 8 and 7)[1] and entitled "*Solanum tuberosum* mRNA for soluble starch synthase II precursor (ssII gene)" and (2) Y10416 entitled "*Solanum tuberosum* mRNA for soluble starch synthase" and providing mRNA for potato starch synthase *I* (thus, *not* the claimed gene). The X87988 record shows a 2631 bp mRNA sequence and references four articles. Two of the four references are from *1995* (prior to the present priority date), one is unpublished, and one is from 2004. Thus, at the time of filing the present application, skilled practitioners had access to publicly available references that provided a sequence for potato starch synthase II. Further, the search did not then and does not now bring up "multitudes of sequences" as suggested by the Examiner in the quote *supra*.

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[1] Applicants will amend the specification to correct this obvious error.

Similarly, an NCBI search for nucleotides sequences of potato SSIII brings up six

core nucleotide sequences and one EST sequence. Of the six core sequences, three are

for *Arabidopsis thaliana*, one is for *Zea mays*, and *two* are for *Solanum tuberosum*. The

two records for the potato are: (1) X94400 entitled "*S. tuberosum* mRNA for soluble-

starch-synthase" and (2) *X95759* entitled "*S. tuberosum* mRNA for starch synthase" and

referenced by *the present specification at page 17*. The X95759 record provides a partial

potato SSIII sequence, while the X94400 record provides a full sequence. Both records

cite two references, which published *prior to* the filing date of the present application.

Again, at the time the present application was being filed, skilled practitioners had easy

access to the potato starch synthase III sequences for the desired isolation and

manipulation. Moreover, there were two, rather than multitudes of, sequences

propounded by the Office in the quote above.

In addition, at the time of filing the application, antisense technology was well-

characterized and could be used to select or even modify desired portions of the

sequences for antisense insertion into potato plants. Similarly, techniques for extracting

and testing the resulting starches were also well-known in the art.

Thus, at the time of filing the present application, a skilled practitioner would

have been able to locate known potato SSII and SSIII sequences and use those and their

functional equivalents with the present methods and plants without undue

experimentation.

C. *Federal Circuit Law Supports Enablement of the*

Present Claims

Moreover, and importantly, Federal Circuit law supports enablement of claims

that recite biological macromolecules without providing a specific sequence for the

macromolecules. For example, in *Falkner v. Inglis*, 448 F.3d 1357, 1365 (Fed. Cir. 2006)

(attached hereto as Evidence Appendix A), the Federal Circuit upheld the decision of the

Board of Patent Appeals and Interferences and confirmed adequacy of enablement of

claims that recited certain genes ***without providing their specific sequences***. The claims

at issue in Falkner were drawn to a vaccine comprising, *inter alia*, a mutant poxvirus

virus, "wherein said mutant virus is a mutant poxvirus and has a genome which ***has an***

inactivating mutation in a viral gene, said viral gene being ***essential*** for the production

of infectious new virus particles." *Id.* at 1360 (emphases added). The application

provided an example of a vaccine of a herpesvirus (a subgenus of the claimed poxvirus)

with its essential gene sequences. *Id.* at 1364-65.

The Federal Circuit held that the claims reciting essential genes of a poxvirus

genus were enabled by: (a) the example showing gene sequences of a herpesvirus species

and (b) by the state of the art. According to the court:

> as of the time of filing of the earliest Inglis application publications in
> professional journals had disclosed the DNA sequence of the poxvirus
> genome along with the locations of the "essential regions." The person of
> ordinary skill in the art would clearly have possessed such knowledge, and
> given the ready accessibility of the journals, the absence of incorporation
> by reference is not problematic. Indeed, "[a] patent need not teach, and
> preferably omits, what is well known in the art." *Id.* at 1365 (citation
> omitted).

Thus, in *Falkner*, the specification provided sequences of a particular species of the claimed genus, whereas the art at the time of filing provided sequences of the genus. Here, even more poignantly, both the examples of the specification and the state of the art provide sequences of the claimed enzyme species and their functional equivalents, namely potato SSII and potato SSIII. As discussed above, the specification refers to Accession Numbers for the sequences used in the experiments, and the professional journal articles published before the priority date disclosed potato SSII and SSIII. Therefore, skilled practitioners would have been able to carry out the presently claimed methods and obtain the presently claimed plants without undue experimentation.

>   D.   *The References Cited by the Examiner Cannot Serve as a*
>
>   *Basis for Nonenablement of the Present Claims*

According to the Advisory Action at page 2:

> **The cited art** shows unpredictability with starch synthase genes when the sequence is altered, and therefore it would be undue experimentation to evaluate all SEQ ID NOs that may be considered potato starch synthase II or potato starch synthase III for their ability in antisense constructs to isolate the starch as broadly claimed in the instant claims. (emphasis added)

Applicants disagree. In the final Office Action, the Examiner cited three references for the proposition that starch synthase genes show unpredictability. The references were: Patron *et al.*, *Plant Physiol.* 130:190-98, 2002 ("Patron," attached hereto as Evidence Appendix B); Edwards *et al.*, *Plant Cell* 14:1767-85, 2002 ("Edwards," attached hereto as Evidence Appendix C); and Salehuzzaman/Shah *et al.*, *Plant Cell Environment* 22:1311-18, 1999 ("Salehuzzaman," attached hereto as Evidence Appendix D).

Patron and Edwards focus on studies and analysis of granule-bound synthase I (GBSSI) in barley and pea, respectively. Patron discusses low-amylose barley mutants of GBSSI and

mentions GBSSI from potato (*Solanum tuberosum*) (at page 196, throughout). Edwards analyzes amylose synthesized by two isoforms of pea GBSSI that were expressed in a potato (at page 1768, second col.). Neither reference discusses methods or plants with nucleic acid sequences encoding potato SSII enzyme and potato SSIII enzyme, according to the present claims. The Office has not shown otherwise. Therefore, neither Patron nor Edwards can serve as a basis for a nonenablement rejection of the present claims. Salehuzzaman is not relevant to the instant claims. Salehuzzaman analyzes cassava GBSSI gene for its ability to produce amylose in amylose-free potato mutants (see, *e.g.*, Abstract). As such, the reference is not relevant to the current claims that recite methods and plants with nucleic acid sequences encoding potato SSII enzyme and potato SSIII enzyme.

At least for the reasons presented above, withdrawal of all enablement rejections is respectfully requested.

II. Written Description Rejections under 35 U.S.C. § 112, First Paragraph

The Office has also maintained the Written Description rejections, as they apply to the entered amended claims. It appears that the Office's arguments cited from the Advisory Action above for Enablement also constitute Written Description rejections. Applicants traverse.

      A.   *The Applicants Were in Possession of the Claimed Embodiments*

              at the Time of Filing the Application

According to the MPEP "to satisfy the written description requirement, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed." MPEP §2163.02.

Here, the claims reciting methods and plants utilizing, *inter alia*, nucleic acid sequences encoding a potato SSII enzyme and a potato SSIII enzyme fully comply with the written description requirement. A skilled practitioner would understand that the Applicants were in possession of the claimed embodiments, because the specification provides examples of transforming plants with nucleic acids encoding potato synthase II and potato synthase III and obtaining starch from such plants (see, *e.g.*, Example 1, sections B) and C) at pages 16-18 of the specification as filed). The Examiner conceded at page 7 of the final Office Action mailed on July 11, 2007, that "the described list [in the specification] only provides examples of functional equivalents of starch synthase II and starch synthase III." Applicants submit that claims 36-43 and 45-52 comply with the written description requirement and respectfully request that all written description rejections be withdrawn.

      B.   *Federal Circuit Law Supports Applicants' Possession of the*

              Claimed Embodiments

Again, Federal Circuit law supports the Applicants' position. As discussed above, the claims at issue in *Falkner* were drawn to a vaccine with mutations in certain genes, whose sequences were not recited. In addition to upholding the enablement of the claims, the Federal

Circuit also held that the claims were fully supported by the specification. The court stated that "it is the binding precedent of this court that *Eli Lilly* does *not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art." *Id.* at 1367 (emphases in the original). Thus, the court held that "where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here, "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences." *Id.* at 1368.

Here, as in *Falker*, scientific journals providing potato SSII and SSIII sequences were available at the time of filing the present application, as discussed above. Moreover, the instant application references the Accession Numbers related to the two enzymes. Therefore, Applicants respectfully submit that the present claims fully meet the written description requirement. Withdrawal of all written description rejections is respectfully requested.

III.   Conclusion

In view of the foregoing, Applicants respectfully submit that no further impediments exist to the allowance of the present claims and, therefore, request an indication of allowability.

This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3). The Notice of Appeal was filed on January 8, 2008, and the Appeal Brief was due by March 8, 2008. A two-month extension of time is hereby requested, thereby making the time of filing May 8, 2008.

The Director is hereby authorized to charge the $510 Brief fee, the $460 extension fee,

and any further appropriate fees, such as fees under 37 C.F.R. §§1.16, 1.17, and 1.21 (except for

the issue fee) that may be required by this paper, and to credit any overpayment, to Deposit

Account No. 50-1283.

Dated: May 8, 2008

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(8)   Claims Appendix

36.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme, and extracting starch from the plant, wherein the starch has a viscosity onset temperature, as judged by viscoamylograph of a 10% w/w aqueous suspension at atmospheric pressure wherein the temperature is reduced by at least about 12° C compared to starch extracted from equivalent, unmodified plants.

37.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme, and extracting starch from the plant, wherein the starch has an endotherm onset temperature, as determined by differential scanning calorimetry, which is reduced by at least about 15° C compared to starch extracted from equivalent, unmodified plants.

38.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme, and extracting starch from the plant, wherein the starch has an endotherm onset temperature, as determined by differential

scanning calorimetry, which is reduced by at least about 17° C compared to starch extracted from equivalent, unmodified plants.

39.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme, and extracting starch from the plant, wherein the starch has an increased amount of starch molecules with a degree of polymerisation of 6-12, as judged by analysis of debranched starch by high performance anion exchange chromatography (HPAEC), compared to starch extracted from equivalent, unmodified plants.

40.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme, and extracting starch from the plant, wherein the starch has a decreased amount of starch molecules with a degree of polymerisation of 15-24, as judged by analysis of debranched starch by HPAEC column, compared to starch extracted from equivalent, unmodified plants.

41.   A method of producing starch comprising stably transforming a potato plant with at least a first and a second antisense heterologous nucleic acid sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic

acid sequence encodes a potato starch synthase III (SSIII) enzyme, wherein the starch has about

a two fold increase in starch molecules with a degree of polymerization of 6-7 and a depletion of

starch molecules with a degree of polymerization between 15-20, as judged by analysis of

debranched starch by HPAEC, compared to starch extracted from equivalent, unmodified plants.

42.   A method of producing starch comprising stably transforming a potato plant with

at least a first and a second antisense heterologous nucleic acid sequence, wherein the first

nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic

acid sequence encodes a potato starch synthase III (SSIII) enzyme, and wherein the starch has an

endotherm onset temperature, as judged by differential scanning calorimetry, of less than about

50° C, compared to starch extracted from equivalent, unmodified plants.

43.   The method of claim 42, wherein the starch extracted from the transformed plant

has an endotherm onset temperature of less than about 44° C.

45.   A plant comprising at least a first and a second heterologous nucleic acid

antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II

(SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII)

enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch

extracted from the plant has a viscosity onset temperature, as judged by viscoamylograph of a

10% w/w aqueous suspension at atmospheric pressure wherein the temperature is reduced by at

least about 12° C compared to starch extracted from equivalent, unmodified plants.

46.   A plant comprising at least a first and a second heterologous nucleic acid antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch extracted from the plant has an endotherm onset temperature, as determined by differential scanning calorimetry, which is reduced by at least about 15° C compared to starch extracted from equivalent, unmodified plants.

47.   A plant comprising at least a first and a second heterologous nucleic acid antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch extracted from the plant has an endotherm onset temperature, as determined by differential scanning calorimetry, which is reduced by at least about 17° C compared to starch extracted from equivalent, unmodified plants.

48.   A plant comprising at least a first and a second heterologous nucleic acid antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch extracted from the plant has an increased amount of starch molecules with a degree of polymerisation of 6-12, as judged by analysis of debranched starch by high performance anion

exchange chromatography (HPAEC), compared to starch extracted from equivalent, unmodified

plants.

49.   A plant comprising at least a first and a second heterologous nucleic acid

antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II

(SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII)

enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch

extracted from the plant has a decreased amount of starch molecules with a degree of

polymerisation of 15-24, as judged by analysis of debranched starch by HPAEC column,

compared to starch extracted from equivalent, unmodified plants.

50.   A plant comprising at least a first and a second heterologous nucleic acid

antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II

(SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII)

enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch

extracted from the plant has about a two fold increase in starch molecules with a degree of

polymerization of 6-7 and a depletion of starch molecules with a degree of polymerization

between 15-20, as judged by analysis of debranched starch by HPAEC, compared to starch

extracted from equivalent, unmodified plants.

51.   A plant comprising at least a first and a second heterologous nucleic acid

antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II

(SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII)

enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch extracted from the transformed plant has an endotherm onset temperature, as judged by differential scanning calorimetry, of less than about 50° C, compared to starch extracted from equivalent, unmodified plants.

52.   A plant comprising at least a first and a second heterologous nucleic acid antisense sequence, wherein the first nucleic acid sequence encodes a potato starch synthase II (SSII) enzyme and the second nucleic acid sequence encodes a potato starch synthase III (SSIII) enzyme and each nucleic acid sequence is operably linked to a promoter, wherein starch extracted from the transformed plant has an endotherm onset temperature, as judged by differential scanning calorimetry, of less than about 44° C, compared to starch extracted from equivalent, unmodified plants.

(9)   **Evidence Appendix**

The Appendix includes four items: A-D.

EVIDENCE APPENDIX A

H

Falko-Gunter Falkner v. Inglis
C.A.Fed.,2006.

United States Court of Appeals,Federal Circuit.
FALKO-GUNTER FALKNER, Georg Holzer, and
Friedrich Dorner, Appellants,
v.
Stephen C. INGLIS, Michael E.G. Boursnell, and
Anthony C. Minson, Appellees.
No. 05-1324.

May 26, 2006.
Rehearing and Rehearing En Banc Denied Aug. 24,
2006.

Background: In interference proceeding, Board of
Patent Appeals and Interferences awarded applicant
priority over patent holder on certain claims on
novel type of vaccine that was comprised of "vector
virus" in poxvirus family. Patent holder appealed.

Holdings: The Court of Appeals, Gajarsa, Circuit
Judge, held that:
(1) disclosure satisfied enablement requirement;
(2) absence of examples involving poxviruses in
patent applications did not render written descrip-
tion inadequate; and
(3) satisfaction of written description requirement
did not require either recitation or incorporation by
reference of genes and their nucleotide sequences.

Affirmed.

West Headnotes

[1] Patents 291 ⭐106(1)

291 Patents
  291IV Applications and Proceedings Thereon
    291k106 Interferences
      291k106(1) k. In General. Most Cited
Cases
Priority in interference proceeding properly could
be decided under old rules, given parties' reliance

on them in filing all motions, oppositions, and
replies in case, which were completed before new
rules took effect.

[2] Patents 291 ⭐99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Spe-
cification. Most Cited Cases
Patent application on novel type of vaccine that was
comprised of "vector virus" in poxvirus family,
which provided detailed example of embodiment
that comprised herpesvirus, rather than poxvirus,
including identity of deleted essential sequences
therein, was adequately enabled, where high level
of skill existed in the art, differences between
herpesviruses and poxviruses were well known,
which would have aided person of ordinary skill in
art in application of lessons of herpesvirus example
in construction of poxvirus vaccines, and publica-
tions in professional journals had disclosed DNA
sequence of poxvirus genome along with locations
of "essential regions."

[3] Patents 291 ⭐99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Spe-
cification. Most Cited Cases

Patents 291 ⭐314(5)

291 Patents
  291XII Infringement
    291XII(C) Suits in Equity
      291k314 Hearing
        291k314(5) k. Questions of Law or
Fact. Most Cited Cases
Under patent law, written description is a question
of fact, judged from the perspective of one of ordin-
ary skill in the art as of the relevant filing date.

[4] Patents 291 ⭐314(5)

291 Patents
    291XII Infringement
        291XII(C) Suits in Equity
            291k314 Hearing
                291k314(5) k. Questions of Law or
Fact. Most Cited Cases
Under patent law, enablement is a question of law
involving underlying factual inquiries.

[5] Patents 291 ☞113(6)

291 Patents
    291IV Applications and Proceedings Thereon
        291k113 Appeals from Decisions of Com-
missioner of Patents
            291k113(6) k. Review on Appeal in Gen-
eral. Most Cited Cases
Court of Appeals applies the standards of the Ad-
ministrative Procedure Act (APA) when reviewing
decisions of the Board of Patent Appeals and Inter-
ferences. 5 U.S.C.A. § 706.

**[6] Administrative Law and Procedure 15A ☞
786**

15A Administrative Law and Procedure
    15AV Judicial Review of Administrative De-
cisions
        15AV(E) Particular Questions, Review of
            15Ak784 Fact Questions
                15Ak786 k. Conflicting Evidence.
Most Cited Cases

Administrative Law and Procedure 15A ☞791

15A Administrative Law and Procedure
    15AV Judicial Review of Administrative De-
cisions
        15AV(E) Particular Questions, Review of
            15Ak784 Fact Questions
                15Ak791 k. Substantial Evidence.
Most Cited Cases
For the purpose of conducting a review of an
agency decision under the Administrative Proced-
ure Act (APA), substantial evidence is defined as
that which a reasonable person might accept as ad-

equate to support a conclusion; it requires an exam-
ination of the record as a whole, taking into account
both the evidence that justifies and detracts from an
agency's opinion. 5 U.S.C.A. § 706.

**[7] Administrative Law and Procedure 15A ☞
791**

15A Administrative Law and Procedure
    15AV Judicial Review of Administrative De-
cisions
        15AV(E) Particular Questions, Review of
            15Ak784 Fact Questions
                15Ak791 k. Substantial Evidence.
Most Cited Cases
For the purpose of conducting a review under the
Administrative Procedure Act (APA), an agency
decision can be supported by substantial evidence,
even where the record will support several reason-
able but contradictory conclusions. 5 U.S.C.A. §
706.

[8] Patents 291 ☞99

291 Patents
    291IV Applications and Proceedings Thereon
        291k99 k. Description of Invention in Spe-
cification. Most Cited Cases
A patent need not teach, and preferably omits, what
is well known in the art.

[9] Patents 291 ☞99

291 Patents
    291IV Applications and Proceedings Thereon
        291k99 k. Description of Invention in Spe-
cification. Most Cited Cases
A patent applicant must convey to those skilled in
the art that, as of the filing date sought, he or she
was in possession of the invention.

[10] Patents 291 ☞99

291 Patents
    291IV Applications and Proceedings Thereon
        291k99 k. Description of Invention in Spe-
cification. Most Cited Cases

No length requirement exists under patent law for a disclosure to adequately describe an invention.

[11] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
Examples are not necessary under patent law to support the adequacy of a written description.

[12] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
Under patent law, the written description standard may be met even where actual reduction to practice of an invention is absent.

[13] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
There is no per se rule under patent law that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

[14] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
Absence of examples involving poxviruses in patent applications on novel type of vaccine that was comprised of "vector virus" in poxvirus family did not render written description inadequate.

[15] Patents 291 ☞99

291 Patents

  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
Under patent law, actual reduction to practice is not required for written description.

[16] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
To the extent that written description requires a showing of possession of the invention, an invention can be "complete" under patent law even where an actual reduction to practice is absent.

[17] Patents 291 ☞99

291 Patents
  291IV Applications and Proceedings Thereon
    291k99 k. Description of Invention in Specification. Most Cited Cases
Where accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences, satisfaction of the written description requirement does not require either the recitation or incorporation by reference of such genes and sequences.

Patents 291 ☞328(2)

291 Patents
  291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
    291k328 Patents Enumerated
      291k328(2) k. Original Utility. Most Cited Cases
5,766,882. Cited as Prior Art.

Patents 291 ☞328(2)

291 Patents
  291XIII Decisions on the Validity, Construction, and Infringement of Particular Patents
    291k328 Patents Enumerated
      291k328(2) k. Original Utility. Most

Cited Cases
5,770,212. Cited.

*1359 John P. Isacson, Heller Ehrman LLP, of
Washington, DC, argued for appellants. With him
on the brief was Paul M. Booth.
Robert G. McMorrow, Jr., Connolly Bove Lodge &
Hutz LLP, of Wilmington, Delaware, argued for ap-
pellee.

Before GAJARSA, Circuit Judge, ARCHER, Seni-
or Circuit Judge and DYK, Circuit Judge.

GAJARSA, Circuit Judge.
This is an appeal from a decision of the Board of
Patent Appeals and Interferences ("Board") in In-
terference No. 105,187, declared on December 24,
2003, between Falkner *et al.*,U.S. Patent No.
5,770,212 ("the Falkner '212 patent") and Inglis *et
al.*,U.S. Application Serial No. 08/459,040 ("the
Inglis '040 application"). The Administrative Patent
Judge (APJ) designated Inglis as the senior party.
On December 29, 2004, the Board issued a final de-
cision, holding that Falkner could not antedate Ing-
lis' September 25, 1990 priority date, and entered
judgment against Falkner on the sole count of the
interference. It ordered that Falkner was not entitled
to claims 1-19 of the Falkner '212 patent. It further
ordered that Inglis was entitled to claims 9, 10, 29
and 30 of the '040 application. Falkner filed a
timely notice of appeal. This Court has jurisdiction
pursuant to 28 U.S.C. § 1295(a)(4)(A) and 35
U.S.C. §§ 141 and 142. For the reasons discussed
below, we affirm the judgment of the Board.

I. BACKGROUND

A. The Invention

Some vaccines against a virus (the "target virus")
incorporate harmless fragments of the target virus's
genetic material into a second virus, called a "viral
vector." When a person is vaccinated, the viral vec-
tor produces harmless fragments of the target virus,

ultimately conferring immunity against it. To pre-
vent the viral vector from itself causing a harmful
infection in *1360 the inoculee, it must be attenu-
ated. Attenuation is achieved by deleting or inactiv-
ating one or more genes responsible for the vector's
growth and infectiousness. However, because the
vaccine is produced by essentially "growing" the
vector virus (accompanied by its inserted target vir-
us gene), attenuation makes it difficult to manufac-
ture the vaccine. The traditional solution to this
problem has been to inactivate genes known as
"inessential" genes. With inessential genes inactiv-
ated, the viral vector is substantially less pathogen-
ic. At the same time, because the vector virus can
still fully reproduce itself, albeit more slowly, the
vaccine can be produced in commercial quantities.
However, the traditional approach carried a disad-
vantage, namely the risk that the vector virus,
though attenuated, could still cause a harmful infec-
tion in the inoculee.

The inventors discovered a way of making vaccines
safer by deleting or inactivating an *essential,* rather
than an inessential, gene from the viral vector's
genome, while at the same time solving the produc-
tion problem by growing the vaccines in cells that
were complementarily modified to produce the ab-
sent essential viral gene product "on behalf of" the
vector virus. Thus, the modified vector virus could
be readily grown in these complementarily-modi-
fied cells, but not in other cells, such as those of an
inoculee.

This approach is applicable to many different kinds
of vector viruses, e.g., adenoviruses, herpesviruses,
poxviruses and retroviruses. The subject matter of
this interference, however, is directed specifically
to vaccines in which the vector virus is a *poxvirus.*
For many vector viruses, there is a risk that vectors
that have been attenuated in essential genes can
"swap" genes with the host cell genome, thereby
reacquiring their deleted genes and reverting to
wild-type virus. This risk can be minimized through
the use of viruses that are "cytoplasmic", meaning
that they are unlikely to enter the cell nucleus. Be-

cause a cell's genes are located in the nucleus, cyto-
plasmic viruses such as poxvirus cannot swap genes
with the cell genome and possibly revert to a viru-
lent wild-type virus.

B. Defining the Count and Assigning Priority

The sole count of the interference was either "[a]
vaccine according to Claim 1 of Falkner's
5,770,212 patent or a vaccine according to Claim
29 of Inglis' 08/459,040 application." Claim 29 of
the Inglis '040 application reads:
A vaccine comprising a pharmaceutically accept-
able excipient and an effective immunizing amount
of a mutant virus, wherein said mutant virus is a
mutant *poxvirus* and has a genome which has an in-
activating mutation in a viral gene, said viral gene
being essential for the production of infectious new
virus particles, wherein said mutant virus is able to
cause production of infectious new virus particles
in a complementing host cell gene expressing a
gene which complements said essential viral gene,
but is unable to cause production of infectious new
virus particles when said mutant virus infects a host
cell other than a complementing host cell; for pro-
phylactic or therapeutic use in generating an im-
mune response in a subject.

(emphasis added)

Claim 1 of the Falkner '212 patent reads:
A vaccine comprising (a) a defective poxvirus that
lacks a function imparted by an essential region of
its parental poxvirus, wherein (i) said defective
poxvirus comprises a DNA polynucleotide encod-
ing an antigen and said DNA polynucleotide is un-
der transcriptional control of a promoter, and (ii)
the function can *1361 be complemented by a com-
plementing source; and (b) a pharmaceutically ac-
ceptable carrier.

[1] The Administrative Patent Judge (APJ) desig-
nated claims 1-19 of the Falkner '212 patent and
claims 9, 10, 29, and 30 of the Inglis '040 applica-
tion as corresponding to the interference count.[FN1]

Both parties sought the benefit of earlier-filed ap-
plications to establish dates of constructive reduc-
tion to practice.[FN2] The ALJ accorded the Inglis
'040 application (filed June 2, 1995) the benefit of
several earlier-filed applications, dating back to
September 25, 1990.[FN3] Likewise, the APJ accor-
ded the Falkner '212 patent (issued June 23, 1998
from an application filed February 21, 1997) the be-
nefit of earlier-filed applications, but these dated
back only to April 29, 1994.[FN4] Consequently, the
APJ designated Inglis as the senior party.

> FN1. Inglis's claim 29 is his broadest
> claim, directed to poxvirus; and claim 30,
> which depends on claim 29, is a poxvirus
> vaccine for mammalian subjects. Claim 9
> is directed to poxvirus but contains some
> additional limitations unrelated to the type
> of virus used; claim 10 depends on claim 9
> and is directed to a single species of
> poxvirus, namely vaccinia virus. Falkner's
> claims 2-10 depend on claim 1. Falkner
> claim 10 is directed to a method of produ-
> cing the vaccine of claim 1, and the re-
> maining method claims depend thereon.

> FN2. Priority in an interference goes to the
> first to invent, but a rebuttable presumption
> exists that the inventors made their inven-
> tions in the chronological order of their ef-
> fective filing dates, namely that the senior
> party invented first, *see* 37 C.F.R. §
> 1.657(a) (2004), and the junior party bears
> the burden of proving otherwise, *see* §
> 1.657(b), such as by proving that she actu-
> ally reduced the invention to practice be-
> fore the constructive filing date (priority
> date) of the senior party, or that she was
> first to conceive and diligently reduced the
> invention to practice, starting from a date
> prior to reduction to practice by the senior
> party. *See* 35 U.S.C. § 112(g) (2000).
> Falkner sought to rely, in part, on an al-
> leged date of conception and beginning of
> reasonable diligence: April 27, 1994.

On September 13, 2004, the "600" rules expired in favor of new rules found at 37 C.F.R. § 41.100 et seq. However, the Board correctly chose to decide the matter under the old rules, given the parties' reliance on them in filing all motions, oppositions, and replies in the case, which were completed before the new rules took effect. *See Singh v. Brake,* 222 F.3d 1362, 1371 (Fed.Cir.2000) (applying a new procedural rule if and only if it did not affect the parties' reliance interests).

> FN3. The Inglis priority applications were U.S. Application Serial No. 08/384,963 ("the Inglis '963 application"), filed February 7, 1995; U.S. Application Serial No. 08/030,073 ("the Inglis '073 application"), filed May 20, 1993; WO/92/05263, PCT/ GB91/01632 ("the Inglis PCT application"), filed September 23, 1991, published in English on April 2, 1992; GB 9104903.1 ("the Inglis 1991 British application"), filed March 8, 1991; and GB 9020799.4 ("the Inglis 1990 British application"), filed September 25, 1990. The Inglis '040 application is a continuation in part of the '963 application, which was in turn a continuation of the Inglis '073 application. The '073 application corresponded to the Inglis PCT application. The Inglis PCT application claimed priority to, and was essentially identical to, the Inglis 1990 and 1991 British applications.

> FN4. The Falkner priority applications were U.S. Application Serial No. 08/616,313 ("the Falkner '313 application") filed March 14, 1996; and U.S. application Serial No. 08/235,392 ("the Faulkner '392 application"), filed April 29, 1994.

C. Board Decision

The specifications of all of Inglis' earlier applications were similar. Although they focused on herpesvirus vectors, they contained several pas-

sages related to poxvirus-based vaccines. Because Falkner believed that these passages did not adequately describe and enable the poxvirus invention, he challenged both Inglis' entitlement to priority as to the count and the patentability of Inglis' corresponding claims. Falkner brought these challenges *1362 in three closely-related preliminary motions before the Board. In each, as the moving party, Falkner carried the burden of proof by a preponderance of the evidence. *See* 37 C.F.R. § 1.637(a); *see also Kubota v. Shibuya,* 999 F.2d 517, 520 n. 2 (Fed.Cir.1993) (explaining that "[t]he term 'burden of proof' ... means the burden to establish the proposition at issue by a preponderance of the evidence").

Falkner brought his first preliminary motion pursuant to 37 C.F.R. § 1.633(a),[FN5] arguing that the claims in Inglis's involved ('040) application that corresponded to the count was unpatentable because they failed to meet the written description requirement of 35 U.S.C. § 112. In support of his argument, he stated, *inter alia,* that (1) the specification of Inglis's '040 application did not identify any essential genes in poxvirus or describe the inactivation of such genes, (2) vaccines based on vaccinia (a type of poxvirus) had not yet been produced, and (3) the bulk of the Inglis specification was directed not to poxviruses but to herpesviruses. The Board denied Falkner's motion, based in part on his failure to address the perceived shortcomings of the '040 claims in light of the specification.

> FN5. On September 13, 2004, the "600" rules expired in favor of new rules found at 37 C.F.R. § 41.100 et seq. However, the Board correctly decided the matter under the old rules, given the parties' reliance on them in filing all motions, oppositions, and replies in the case, which were completed before the new rules took effect. *See Singh v. Brake,* 222 F.3d 1362, 1371 (Fed.Cir.2000) (applying a new procedural rule if and only if it did not affect the parties' reliance interests); *see also Brown*

v. *Barbacid,* 436 F.3d 1376, 1379 n. 1 (Fed.Cir.2006) (holding that the Board did not err in applying the old rules "under which this case was decided").

Second, Falkner moved pursuant to 37 C.F.R. §§ 1.633(g) & 1.637(g) to deny Inglis the priority benefit of his earlier applications, arguing that they did not sufficiently describe and enable the claims in question.[FN6] Falkner argued that without the benefit of these applications Inglis would be unable to establish constructive reduction to practice earlier than Falkner. Falkner would win priority as to the count, and Inglis' corresponding claims would be unpatentable. In support of his motion, Falkner alleged deficiencies in Inglis' benefit specifications similar to those raised in his first motion. The Board carefully articulated the legal standard, correctly explaining that "benefit with respect to priority in an interference is granted with respect to *counts* not *claims* " and that "[a]ll that is necessary for a party to be entitled to benefit of an earlier filed application for priority purposes is compliance with 35 U.S.C. § 112 with respect to at least one embodiment within the scope of the count." *Board Op.* at 7 (citing *Hunt v. Treppschuh,* 523 F.2d 1386, 1389 (CCPA 1975) (holding that where a "parent application is relied upon as a prior constructive reduction to practice[,] .... the § 112, first paragraph requirements need only be met for an embodiment within the count")). After careful review of the record, the Board held that Falkner had failed to meet his burden of proof.

FN6. Falkner did not argue lack of enablement with respect to the Inglis '963 patent because he believed that the teachings of the Falkner '392 patent, filed in 1994, would have enabled the subsequent '963 patent.

Third, Falkner moved for judgment pursuant to 37 C.F.R. § 1.633(a) that the claims in Inglis' involved ('040) application that corresponded to the count were anticipated and therefore unpatentable. He argued that because Inglis' earlier applications had

failed to adequately describe and enable the full scope of his current claims, the current claims could not be accorded the benefit of 35 U.S.C. § 120 for the *1363 purpose of antedating patent-defeating prior art.[FN7] The Board explained that 35 U.S.C. §§ 119 & 120 require benefit applications to comply with § 112, first paragraph, with respect to the *full scope* of what a party now claims, rather than with respect to merely one embodiment within the scope of the interference count. After carefully considering the written description and enablement issues, the Board denied the motion. As a result of the denial of Falkner's several motions, Inglis remained the senior party, and the Board ordered judgment as to the subject matter of the count in favor of Inglis.

FN7. Here, Falkner points to his own U.S. Pat. No. 5,766,882 ("the '882 patent' "), issued in March 1995, as the patent-defeating prior art.

D. Issue and Standard of Review

[2] On appeal, Falkner essentially reiterates the arguments that he made before the Board. While we recognize that each of these three arguments is distinct, they are nonetheless all related, and under the facts of this particular case, we need only to resolve the following common issue: whether the Inglis benefit applications adequately describe and enable a poxvirus-based vaccine. Falkner also argues that the Board committed other errors, such as initially designating Inglis as the senior party and failing to afford Falkner an opportunity for briefing prior to making this designation. These arguments lack merit, and we shall not further discuss them. We turn, therefore, to the central issues in this case: written description and enablement.

[3][4] Written description is a question of fact, judged from the perspective of one of ordinary skill in the art as of the relevant filing date. *See Vas-Cath, Inc. v. Mahurkar,* 935 F.2d 1555, 1563-64 (Fed.Cir.1991). Enablement is a question of law involving underlying factual inquiries. *See Gen-*

entech, Inc. v. Novo Nordisk A/S, 108 F.3d 1361, 1365 (Fed.Cir.1997); see also In re Wands, 858 F.2d 731, 737 (Fed.Cir.1988) (holding that whether undue experimentation is required is a "conclusion reached by weighing many factual considerations.... includ[ing] (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.").

[5] This court applies the standards of the Administrative Procedure Act ("APA") in reviewing decisions of the Board. See Dickinson v. Zurko, 527 U.S. 150, 152, 119 S.Ct. 1816, 144 L.Ed.2d 143 (1999) (holding that 5 U.S.C. § 706 governs our review of PTO appeals). Accordingly, we will set aside actions of the Board if they are arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law, and we set aside factual findings that are unsupported by substantial evidence. See In re McDaniel, 293 F.3d 1379, 1382 (Fed.Cir.2002) (citing 5 U.S.C. § 706); see also In re Sullivan, 362 F.3d 1324, 1326 (Fed.Cir.2004) (substantial evidence review of factual findings). We review questions of law de novo. See Rapoport v. Dement, 254 F.3d 1053, 1058 (Fed.Cir.2001).

[6][7] Substantial evidence is defined as that which a reasonable person might accept as adequate to support a conclusion. See In re Zurko, 258 F.3d 1379, 1384 (Fed.Cir.2001). It requires an examination of the record as a whole, taking into account both the evidence that justifies and detracts from an agency's opinion. See *1364In re Gartside, 203 F.3d 1305, 1312 (Fed.Cir.2000) . An agency decision can be supported by substantial evidence, even where the record will support several reasonable but contradictory conclusions. See id.; see also In re Jolley, 308 F.3d 1317, 1320 (Fed.Cir.2002).

II. DISCUSSION

A. Contents of the Inglis Priority Applications

The claims that correspond to the count of the interference are directed to a novel type of vaccine that is comprised of a "vector virus" in the poxvirus family. Conceptually, poxviruses are a "subgenus" of viruses that includes the "species" vaccinia. All of the prior Falkner applications described poxvirus vaccine vectors in detail, and to the exclusion of other types of vaccine vectors (e.g., herpesvirus vaccine vectors). These applications provided five detailed working examples regarding the preparation and use of vaccines from defective poxviruses. They also described the use of a particular species of poxvirus vaccine vector, namely vaccinia virus.

In contrast, the Inglis applications described vaccine vectors in general, and then focused on the subgenus of herpesviruses, for which they provided a detailed example. Nevertheless, at least three passages discussed the poxvirus invention and specifically mentioned "vaccinia virus." [FN8] For example, after introducing the concept of vaccine vectors, the specification states that "[t]ypically members of the pox virus family, e.g. vaccinia virus, are used as vaccine vectors." [FN9] The specification later discusses the deletion of essential genes from vaccine vector genomes, noting that the "invention can be applied to any virus where one or more essential gene(s) can be identified and deleted from or inactivated within the virus genome" (emphasis added). Moreover, it provides that "the virus may comprise an orthopox virus, for example, vaccinia virus, which may comprise a heterologous sequence encoding an immunogen derived from a pathogen." Finally, it reads:

> FN8. We recognize that the Inglis applications do not describe any actual reduction to practice of a poxvirus vaccine. See Carroll Declaration (stating that the '040 application did not contain any discussion of the "actual creation of the recited 'mutant poxvirus' " and that the application in fact stated that "a vaccinia virus with a deletion

in an essential gene had not been produced."). As we discuss below, however, an actual reduction to practice is unnecessary to satisfy the written description requirement.

FN9. Because of the substantial similarity in the specifications of all of the Inglis benefit applications, we shall refer in this opinion to representative passages from the earliest of the applications, the Inglis 1990 British application.

For example vaccinia virus, a poxvirus, can carry and express genes from various pathogens, and it has been demonstrated that these form effective vaccines when used in animal experimental systems. The potential for use in humans is vast, but because of the known side effects associated with the widespread use of vaccinia as a vaccine against smallpox, there is reluctance to use an unmodified vaccine in humans. There have been attempts to attenuate vaccinia virus by deleting non-essential genes such as the vaccinia growth factor gene... However, such attenuated viruses can still replicate in vivo, albeit at a reduced level. No vaccinia virus with a deletion in an essential gene has yet been produced, but such a virus, deleted in an essential gene as described above, with its complementing cell for growth, would provide a safer version of this vaccine.

The application provides a detailed example of an embodiment that comprised not a poxvirus, but a *herpesvirus,* including*1365 the identity of the deleted essential sequences therein. Nevertheless, for the reasons discussed below, we find no error in the Board's determinations on the adequacy of written description and enablement in the various Inglis disclosures.

B. Enablement

Because the adequacy of the disclosure is judged from the perspective of one of ordinary skill in the

art, we start our review of the Board's decision by noting that the parties stipulated to a high level of skill in the art. They defined the skilled artisan as having 5-10 years experience creating recombinant poxvirus, as being familiar with the poxvirus literature, the use of poxvirus as a vector for the expression of heterologous genes, and having the "needed technical skill to practice the experimentation described in the scientific literature relating to recombinant virus, including poxvirus." The Board agreed with the parties' stipulation as to level of skill.

The Board did not err in finding Inglis' claims to be enabled as a matter of law, in light of its articulated underlying factual findings. In support of its conclusion, it noted that "there is extensive disclosure of the selection of an essential gene, its deletion or inactivation and the production of a mutated virus with said deleted or inactivated gene, albeit for herpesvirus." Moreover, because the differences between the herpesviruses and poxviruses were well known, this would have aided the person of ordinary skill in the art in her application of the lessons of the herpesvirus example in the construction of poxvirus vaccines. The Board observed that "the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be 'undue' in this art. Indeed, great expenditures of time and effort were ordinary in the field of vaccine preparation." Thus, the Board found the Inglis applications to be enabling.

[8] Reviewing the Board's legal conclusion of enablement, as based on its underlying findings of fact, we cannot say that the Board erred. With respect to a skilled artisan's ability to identify "essential" poxvirus genes, as discussed below we note that there was undisputed testimony that as of the time of filing of the earliest Inglis application publications in professional journals had disclosed the DNA sequence of the poxvirus genome along with the locations of the "essential regions." The person of ordinary skill in the art would clearly

have possessed such knowledge, and given the ready accessibility of the journals, the absence of incorporation by reference is not problematic. Indeed, "[a] patent need not teach, and preferably omits, what is well known in the art." *Spectra-Physics, Inc. v. Coherent, Inc.,* 827 F.2d 1524, 1534 (Fed.Cir.1987).

C. Written Description

[9] On appeal to this court, Falkner essentially reargues the positions on written description that he took before the Board. Although the Board erred in its articulation of the written description standard, that error is harmless. The Board held that "an actual possession standard is *not* required."(emphasis added). But our precedent clearly establishes that "[t]he applicant must ... convey to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention." *Vas-Cath, Inc. v. Mahurkar,* 935 F.2d 1555, 1563-64 (Fed.Cir.1991). Nonetheless, we conclude there is no need for remand because the undisputed testimony supports the Board's ultimate conclusion.

[10] As noted above, the Board found several passages in the Inglis '040 application (and in the benefit applications) that were directed to poxvirus. No length requirement exists for a disclosure to adequately*1366 describe an invention. *See In re Hayes Microcomputer Prods., Inc. Patent Litig.,* 982 F.2d 1527, 1534 (Fed.Cir.1992) ("[T]he adequacy of the description of an invention depends on its content in relation to the particular invention, not its length."). Furthermore, the testimony of Inglis' expert, Dr. Boursnell, established that the articles describing essential genes for poxvirus were well-known in the art. Dr. Boursnell testified that "the skilled person would have been readily able to choose an essential vaccinia gene" based on references that have been publicly available since 1990. The testimony of Falkner's expert, Dr. Carroll, did not refute this claim.

[11][12][13] The parties also dispute several as-

pects of our law of written description, which we now address. We conclude that the Board applied correct law. Specifically, we hold, in accordance with our prior case law, that (1) examples are not necessary to support the adequacy of a written description (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no *per se* rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

1. Examples Are Not Required

[14] First, it is clear that the absence of examples involving poxviruses in the Inglis applications does not render the written description inadequate. As we explained in *LizardTech, Inc. v. Earth Resource Mapping, PTY, Inc.:*

A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before. Placed in that context, it is unnecessary to spell out every detail of the invention in the specification; only enough must be included to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation.

424 F.3d 1336, 1345 (Fed.Cir.2005) (citing *Union Oil Co. v. Atl. Richfield Co.,* 208 F.3d 989, 997 (Fed.Cir.2000); *In re GPAC Inc.,* 57 F.3d 1573, 1579 (Fed.Cir.1995)).

2. Actual Reduction to Practice Is Not Required

[15][16] As we explained in *Capon v. Eshhar,* "[t]he 'written description' requirement implements the principle that a patent must describe the technology

that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed." 418 F.3d 1349, 1357 (Fed.Cir.2005). The Board was correct, however, not to view as dispositive that Inglis had not actually *produced* a poxvirus vaccine,[FN10] because an actual reduction to practice is not required for written description.[FN11] *See* *1367 *Univ. of Rochester v. G.D. Searle & Co.,* 358 F.3d 916, 926 (Fed.Cir.2004) ("We of course do not mean to suggest that the written description requirement can be satisfied only by providing a description of an actual reduction to practice. Constructive reduction to practice is an established method of disclosure ...."). *Rochester,* moreover, is consistent with Supreme Court precedent. In the context of interpreting 35 U.S.C. § 102(b), the Court held that "[t]he word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' " *Pfaff v. Wells Elecs.,* 525 U.S. 55, 66, 119 S.Ct. 304, 142 L.Ed.2d 261 (1998). It then proceeded to make clear that although "reduction to practice ordinarily provides the best evidence that an *invention* is complete.... it does not follow that proof of reduction to practice is necessary in every case." *Id.* (emphasis added).FN12 Thus, to the extent that written description requires a showing of "possession of the *invention,*" *Capon,* 418 F.3d at 1357 (emphasis added), *Pfaff* makes clear that an invention can be "complete" even where an *actual* reduction to practice is absent.[FN13] The logical predicate of "possession" is, of course, "completeness."

> FN10. The Inglis specifications stated that "[n]o vaccinia virus with a deletion in an essential gene has yet been produced, but such a virus, deleted in an essential gene as described above, with its complementing cell for growth, would provide a safer version of this vaccine."

> FN11. The Board believed that Falkner's

expert, Dr. Carroll, had premised his opinions on the misunderstanding that actual reduction to practice was required to prove written description, and it discredited his expert opinion.

> FN12. Similarly, this court has carefully explained the relationship between written description and possession, explaining that a showing of possession is not necessarily sufficient to demonstrate the adequacy of written description. *See, e.g., Enzo Biochem, Inc. v. Gen-Probe Inc.,* 296 F.3d 1316, 1330 (Fed.Cir.2002) ("[P]roof of a reduction to practice, absent an adequate description in the specification of what is reduced to practice, does not serve to describe or identify the invention for purposes of § 112, P 1. As with 'possession,' proof of a reduction to practice may show priority of invention or allow one to antedate a reference, but it does not by itself provide a written description in the patent specification.").

> FN13. In contrast to reduction to practice, conception *is* a prerequisite to an adequate written description. *See Fiers v. Sugano,* 984 F.2d 1164, 1171 (Fed.Cir.1993) ("[O]ne cannot describe what one has not conceived.").

3. Recitation of Known Structure Is Not Required

[17] Falkner argues, *inter alia,* that the Inglis specifications do not adequately describe the poxvirus invention, in light of *Eli Lilly,* because they do not describe the "essential regions" of any poxvirus. 119 F.3d 1559. We note, in addition, that Inglis did not attempt to incorporate by reference any literature that described the DNA sequence of the poxvirus genome and the locations of the "essential regions." However, it is the binding precedent of this court that *Eli Lilly* does *not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must

always recite the gene or sequence, regardless of whether it is known in the prior art. *See Capon,* 418 F.3d at 1357 ("None of the cases to which the Board attributes the requirement of total DNA reanalysis, i.e., *Regents v. Lilly, Fiers v. Revel, Amgen,* or *Enzo Biochem,* require a re-description of what was already known."). Thus, "[w]hen the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh." *Id.* at 1358. Rather, we explained that:

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the *1368 state of knowledge in the field and differences in the predictability of the science.

Id. at 1357.

Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention. As we stated in *Capon,* "[t]he 'written description' requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution." *Id.* at 1358. Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification. Accordingly we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their

nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference [FN14] (where permitted) of such genes and sequences.

> FN14. Here, the patentee did not attempt incorporation by reference. Where, of course, certain material that is not present in the specification is deemed nonessential to the satisfaction of the written description requirement, the issue of proper incorporation by reference *vel non* is irrelevant.

In conclusion, having reviewed the decision of the Board, we can discern no error in its conclusion that the disclosures relied upon by Inglis for priority purposes adequately described and enabled the invention directed to poxvirus, there being substantial evidence to support these findings. Consequently, we hold that the Board's award of priority to Inglis was proper.

AFFIRMED

No costs.

C.A.Fed.,2006.
Falko-Gunter Falkner v. Inglis
448 F.3d 1357, 79 U.S.P.Q.2d 1001

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EVIDENCE APPENDIX B

The Altered Pattern of Amylose Accumulation in the Endosperm of Low-Amylose Barley Cultivars Is Attributable to a Single Mutant Allele of Granule-Bound Starch Synthase I with a Deletion in the 5'-Non-Coding Region[1]

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Reasons for the variable amylose content of endosperm starch from *waxy* cultivars of barley (*Hordeum vulgare*) were investigated. The mature grains of most such cultivars contain some amylose, although amounts are much lower than in wild-type cultivars. In these low-amylose cultivars, amylose synthesis starts relatively late in grain development. Starch granules in the outer cell layers of the endosperm contain more amylose than those in the center. This distribution corresponds to that of granule-bound starch synthase 1 (GBSSI), which is more severely reduced in amount in the center of the endosperm than in the outer cell layers, relative to wild-type cultivars. A second GBSSI in the barley plant, GBSSIb, is not detectable in the endosperm and cannot account for amylose synthesis in the low-amylose cultivars. The change in the expression of GBSSI in the endosperm of the low-amylose cultivars appears to be due to a 413-bp deletion of part of the promoter and 5'-untranslated region of the gene. Although these cultivars are of diverse geographical origin, all carry this same deletion, suggesting that the low-amylose cultivars have a common *waxy* ancestor. Records suggest a probable source in China, first recorded in the 16th century. Two further families of *waxy* cultivars have no detectable amylose in the endosperm starch. These amylose-free cultivars were selected in the 20th century from chemically mutagenized populations of wild-type barley. In both cases, 1-bp alterations in the GBSSI gene completely eliminate GBSSI activity.

The aim of this work was to investigate the reported variation in the amylose content of starch from the endosperm of *waxy* mutants of barley (*Hordeum vulgare*). Amylose is synthesized by granule-bound starch synthase I (GBSSI), an isoform of starch synthase of approximately 60 kD. GBSSI is encoded at the *Waxy* loci in cereals. In most cereal species, *waxy* mutants lack any detectable amylose in the starch of the endosperm. The major exception is barley, in which *waxy* mutant cultivars are reported to have between 0% and 13% amylose in their starch. For most such low-amylose cultivars, endosperm starch is reported to contain between 0.4% and 9% amylose (Banks et al., 1970; Morrison et al., 1986; McDonald et al., 1991; Song and Jane, 2000), but starch from a few cultivars has undetectable amylose (barley cv Yon M Kei, Ishikawa et al., 1995; barley cv CDC Alamo [line SB94794], Bhatty and Rossnagel,

1997). In this paper, the *waxy* mutants with detectable amylose will be referred to as low-amylose cultivars and those with undetectable amylose will be referred to as amylose-free.

In one low-amylose barley line (SW7142-92), the residual amylose has been shown to be concentrated in the outer layer of cells of the endosperm. Starch in the cells in this subaleurone layer stained blue-black with iodine solution, whereas that in the remainder of the endosperm stained red. The amylose contents of starch from tissues dissected from the outer and innermost parts of the grains of this cultivar were 8.6% and 2.2%, respectively (Oscarsson et al., 1997; Andersson et al., 1999).

Two possible explanations for the wide variation in amylose content of the starch of barley *waxy* mutants are suggested by recent studies of GBSSI. First, it has been shown that wheat (*Triticum aestivum*) possesses two isoforms of GBSSI with different spatial distributions in the plant. In developing wheat grains, one GBSSI isoform accounts for amylose synthesis in the endosperm and a second accounts for much of the amylose synthesis in the pericarp, aleurone, and embryo (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000). The pea (*Pisum sativum*) plant also has two, differently expressed iso-
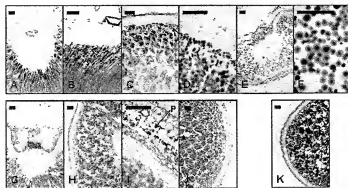
Figure 1. A through K, Developing endosperm and starch from *waxy* barley mutants. For endosperm sections, whole grains were fixed in formaldehyde, embedded in wax, sectioned, and stained with iodine solution. All samples were taken from grain of 50 to 70 mg fresh weight (starting to turn yellow) except for those in E and G, which were from grain of approximately 20 mg fresh weight. A through F, Low-amylose cultivars. G through H, Amylose-free cultivars. K, Wild-type cultivar. P, Pericarp. A, Barley cv Iyatomi Mochi. B, Barley cv Iyatomi Mochi. C, Barley cv Waxy Oderbrucker. D, Barley cv SB85750. E, Barley cv Iyatomi Mochi, young grain. F, Starch extracted from barley cv Waxy Hector. G, Barley cv Yon M Kei. H, Barley cv CDC Alamo. I, Barley cv CDC Alamo, young grain. J, Barley cv Arizona Hulless Waxy. K, Barley cv Shikoku Hadaka.

forms of GBSSI (Denyer et al., 1997). It is likely that in barley, as in wheat, two isoforms of GBSSI are present and expressed in different tissues. However, if in barley the isoform expressed primarily in other parts of the plant was also expressed in the endosperm of barley in addition to the endosperm-specific isoform of GBSSI, then loss of either GBSSI could result in a low-amylose content. Loss of both forms of GBSSI from the endosperm would result in amylose-free starch.

Second, several independently derived waxy cultivars of barley have been shown to possess identical deletions in a GBSSI gene expressed in the endosperm—the only GBSSI gene thus far identified in barley. The deletion overlaps a TATA box, and reverse transcriptase (RT)-PCR failed to reveal any mRNA for GBSSI in endosperm of the mutant cultivars (Drescher et al., 2000). However, the data do not rule out the possibility that the deletion drastically reduces but does not eliminate expression of the gene. Thus, the presence of amylose in the endosperms of the low-amylose cultivars of barley might be explained by the widespread occurrence of a mutation that reduces but does not entirely eliminate expression of the gene encoding endosperm GBSSI.

To discover which, if either, of these explanations is correct, we have examined the occurrence and distribution of amylose and GBSSI protein(s), and the nature and expression of genes encoding GBSSI in the developing grains of low-amylose and amylose-free mutants of barley.

RESULTS

The Distribution of Amylose in the Endosperm

Iodine-stained sections of developing grain revealed great variation between *waxy* cultivars in the proportion of endosperm cells that contained significant amounts of amylose. In the amylose-free barley cv Yon M Kei and cv CDC Alamo, we observed no blue-staining granules at any stage of development (Fig. 1, G–I). Barley cv Arizona Hulless Waxy, a parent of CDC Alamo, also had no blue-staining granules (Fig. 1J); therefore, we consider it to be an amylose-free cultivar. In the low-amylose barley cv Iyatomi Mochi, cv Waxy Oderbrucker, cv Waxy Hector, and line SB85750 (the other parent of CDC Alamo; Bhatty and Rossnagel, 1997), no blue-staining granules were present in young endosperm (from grain up to about 20 mg fresh weight), but blue-staining granules appeared in outer cells of the endosperm during the later part of development (Fig. 1, A–F). In some of the low-amylose cultivars, blue-staining granules in the endosperm were largely confined to cells immediately adjacent to the groove (inside the basal endosperm transfer cell layer; Olsen et al., 1999), whereas in others, blue-staining starch granules were present in cells at the outer edge of the endosperm all around the grain (Fig. 1, A–D). In most cases, blue-staining granules were not confined to a single layer of cells. There was a gradation from the outer edge of the endosperm of cells with blue staining, through cells in which the peripheral region of the granule stained red and the core stained blue, to cells in which the entire granule stained red. In barley cv Waxy Hector, a low-amylose cultivar reported to have up to 8% amylose in its starch (Morrison et al., 1986), granules containing some amylose were present from early in development (in grains of less than 20 mg fresh weight). In more mature endosperms of barley cv Waxy Hector, most of the granules stained either completely or partly blue with iodine solution (Fig. 1F).

In all of the cultivars, including those with no amylose in the endosperm, starch in the pericarp
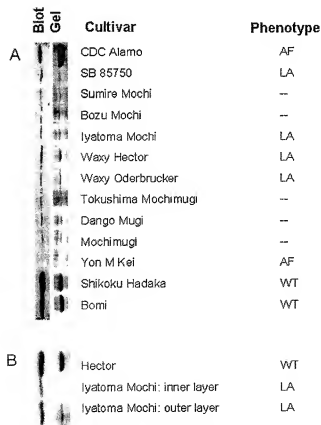
(Fig. 1I) and in the embryo (not shown) stained blue with iodine at all of the developmental stages at which it was present.

The Presence of GBSSI Protein in the Endosperm

In all of the low-amylose cultivars, the starch contained a protein of approximately 60 kD, immunologically related to the GBSSI present in wild-type barley but present in very much lower concentrations than in wild-type starch (Fig. 2A). In the amylose-free *waxy* barley cv Yon M Kei, as reported previously (Ishikawa et al., 1995), no 60-kD protein was detectable (Fig. 2A). However, in the amylose-free barley

Figure 2. A and B, Presence of GBSSI-like proteins in starch from the developing endosperm of barley. After gelatinization by boiling in SDS-containing buffer, samples of starch from developing endosperms (from grains of approximately 50 mg fresh weight) were subjected to electrophoresis on 7.5% (w/v) SDS-polyacrylamide gels. Gels were either stained with Coomassie Brilliant Blue (right) or blotted onto nitrocellulose (left). Blots were developed with serum containing antibodies against pea embryos at a dilution of 1:2,000 (v/v; A) or 1:750 (v/v; B). A, Gel and blot of starch granule-bound proteins from whole endosperms. The phenotypes of the cultivars with respect to their amylose contents are indicated. WT, Wild type. LA, Low amylose. AF, amylose free. B, Gel and blot of starch from dissected outer layers and inner part of the endosperm of the low-amylose barley cv Iyatomi Mochi, and from whole endosperm of the wild-type barley cv Hector.

cv CDC Alamo, the amount of the 60-kD protein was very similar to that in representative wild-type cultivars. Measurements of starch synthase activity associated with granules in developing endosperm of barley cv CDC Alamo suggested that most or all of this GBSSI protein was inactive. The activity was comparable with or lower than that of other *waxy* cultivars, and only about 10% of that of wild-type cultivars (data not shown). Much or all of this residual activity is likely to be due to isoforms of starch synthase other than GBSSI (Hylton et al., 1995).

To study the distribution of the GBSSI-like 60-kD protein in the endosperms of a low-amylose cultivar, the outer layers were dissected away from the inner part of the maturing endosperm of barley cv Iyatomi Mochi. Starch extracted from the outer layers contained considerably more of the 60-kD protein than starch from the inner part (Fig. 2B).

Comparison of GBSSI Isoforms

To discover whether in barley, as in wheat, there is a second form of GBSSI, we searched for a barley expressed sequence tag (EST) similar to the non-endosperm form of GBSSI in wheat (GBSSII; Vrinten and Nakamura, 2000). A barley EST (accession no. AL508718) was identified and used to clone a cDNA of a second form of barley GBSSI, which we called GBSSIb (submitted to GenBank; accession no. AF486521). The predicted mature GBSSIb protein shares 96.4% identity with wheat GBSSII and 65.3% identity with barley GBSSI. Thus, in barley, as in wheat, there are two forms of GBSSI that are similar in amino acid sequence but differ particularly at the N termini of the mature proteins (Table I). To investigate whether GBSSIb is expressed in the outer cell layers of the endosperm of barley, we compared the predicted protein sequences of the two isoforms of GBSSI with protein sequences obtained experimentally from starch from wild-type and *waxy* barley endosperms.

GBSSI proteins in wild-type and low-amylose cultivars were compared by matrix-assisted laser-desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) and by N-terminal sequencing. MALDI-TOF MS was performed on tryptic digests of GBSSI purified from starch from the wild-type barley cv Hector and the low-amylose barley cv Waxy Hector and Iyatomi Mochi. For barley cv Hector, 13 peptides were identified that accounted for 29% of the amino acids in the mature GBSSI protein. For barley cv Waxy Hector, 16 peptides were identified that accounted for 32% of the amino acids in GBSSI. For barley cv Iyatomi Mochi, 14 peptides were identified that accounted for 32% of the amino acids in GBSSI. For all three samples, the best match of peptide masses obtained was to the amino acid sequence predicted from the barley GBSSI cDNA sequence (accession no. X07932; Rohde et al., 1988). These results are consistent with the idea that the

Table I. *Comparison of the N-terminal sequences of GBSSI from wheat and barley*

Sequences for Waxy Oderbrucker (low amylose) and Shikoku Hadaka (wild type) were obtained experimentally from GBSSI proteins purified from starch granules extracted from developing barley endosperm. The N-terminal sequence of the mature barley GBSSIb was predicted from the cDNA sequence (AF486521) using TargetP (Emanuelsson et al., 2000). The other sequences were reported previously (barley GBSSI, Vogelsanger Gold, Rohde et al., 1988; wheat GBSSI, Ainsworth et al., 1993; and wheat GBSSII, Nakamura et al., 1998). —, Identification was not possible. Parentheses indicate uncertainty.

Cultivar							N-Terminal Sequence						
Waxy Oderbrucker		A	T	G	S/(N)	—	M	N	L	V	F	(V)	G
Shikoku Hadaka		A	T	G	S	G	M	N	L	V	F	V	G
Vogelsanger Gold		A	T	G	S	G	M	N	L	V	F	V	G
Wheat GBSSI	A	T	G	S	G	G	M	N	L	V	F	V	G
Wheat GBSSII				S	T	G	M	P	I	I	F	V	A
Barley GBSSIb				S	T	G	M	P	I	I	F	V	A

GBSSI-like protein in the low-amylose barley cv Iyatomi Mochi and cv Waxy Hector is the product of the same gene that encodes the endosperm GBSSI in wild-type barley.

Protein sequencing revealed that the N-terminal 12 amino acids of the GBSSI protein from starch from the outer part of the endosperm of the low-amylose barley cv Waxy Oderbrucker matched the sequence of the GBSSI protein from the endosperm of the wild-type barley cv Shikoku Hadaka and cv Vogelsanger Gold (Table I). The sequences of these proteins were also very similar to that of the GBSSI expressed in wheat endosperm (Ainsworth et al., 1993; Taira et al., 1995). They differed considerably from the N-terminal sequences of the nonendosperm form of GBSSI in wheat (GBSSII) and barley (GBSSIb). These data again suggest strongly that the GBSSI in the endosperm of the low-amylose *waxy* cultivars of barley is the same protein as that in the endosperm of wild-type barley, rather than a different isoform expressed primarily in other parts of the plant.

Mutations in the GBSSI Gene of Waxy Barleys

To provide further evidence about the identity of the GBSSI in the endosperm of *waxy* barley cultivars, we cloned and sequenced the cDNA encoding GBSSI and 1 kb of the promoter region of the GBSSI gene from a wild-type cultivar, barley cv Oderbrucker, and from several low-amylose (Waxy Oderbrucker, Iyatomi Mochi, and SB85750) and amylose-free (Yon M Kei and CDC Alamo) lines and cultivars. The sequence of GBSSI obtained from barley cv Oderbrucker (accession no. AF486514) was almost identical to that of the wild-type barley cv Vogelsanger Gold, published earlier (accession no. X07931). The 5′-untranslated region (UTR) of these wild-type alleles includes intron 1 (Fig. 3), and the region upstream of the 5′-UTR contains a predicted transcription complex-binding site (TATA box) 43 bp upstream of the transcription start site (Rohde et al., 1988).

The GBSSI sequences for all of the low-amylose cultivars were very similar to one another and different from the sequences from wild-type barley in two main respects. First, in the GBSSI alleles from the low-amylose cultivars, there was a 413-bp deletion in the promoter and 5′-UTR including the TATA box, the start of transcription, and part of intron 1 (Fig. 3). Second, there was also a 15-bp insertion in exon 1 that does not cause a frame shift but results in the addition of five extra amino acids to the transit peptide of the protein. To discover more about the distribution of this 15-bp insertion among barley cultivars, we sequenced the same region from barley cv Shikoku Hadaka, from which the *waxy* barley cv Yon M Kei was derived. The 15-bp insertion was present in the GBSSI allele in this cultivar (Table II, column 1). Thus, the insert represents allelic variation that has little or no impact upon amylose content: It cannot be responsible for the low-amylose phenotype. We conclude that the reduction in amylose content in low-amylose cultivars is probably due to the 413-bp deletion that is common to all of the cultivars of this type that we have examined.

The GBSSI sequence from the amylose-free barley cv CDC Alamo was identical to that of the wild-type barley cv Vogelsanger Gold except for one base substitution (T instead of A) at position 860 in barley cv CDC Alamo. This is predicted to result in the substitution of the aliphatic amino acid Val (Gly) for the acidic amino acid Asp (Asp). Asp is conserved in this position in all of the other GBSSI alleles of barley (Table II) and in GBSSI from a wide range of other species (data not shown). This single base change in barley cv CDC Alamo is likely to be the cause of the observed production of an inactive GBSSI protein in this cultivar (see above). Thus, it defines an Asp that is essential for GBSSI activity. The sequence of GBSSI from the amylose-free barley cv Yon M Kei also contained a single base substitution compared with wild-type sequences, at position 580 (T instead of C). This is predicted to create a stop codon, and thus is likely to be responsible for the complete lack of GBSSI protein that was observed in this mutant.
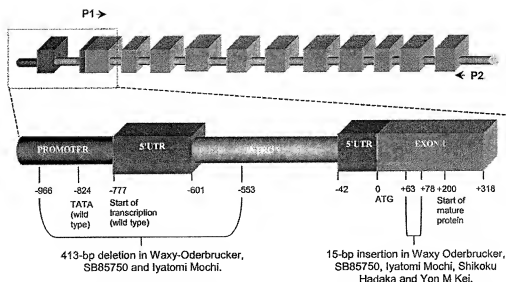
Figure 3. Diagrammatic representations of the gene structure of GBSSI. Upper diagram, The entire GBSSI gene. Blocks represent the 5'-UTR and the exons. Lower diagram, the 5'-UTR and exon 1 expanded to show the 413-bp deletion and 15-bp insertion. The sequences for the promoter and 5'-UTRs of GBSSI have been submitted to GenBank (accession nos.: barley cv Oderbrucker, AF486508; barley cv Waxy Oderbrucker, AF486509; barley cv Iatoma Mochi, AF486510; SB85750, AF486511; barley cv CDC Alamo, AF486512; and barley cv Yon M Kei, AF486513).

In addition to the 15-bp insertion in the GBSSI gene already mentioned (Fig. 3), there are other sequence differences between the GBSSI alleles for which we have complete sequences. These are summarized in Table II. These minor differences are not correlated with amylose content and therefore are unlikely to contribute to the *waxy* phenotype. However, they represent at least some of the allelic variation in GBSSI that exists within cultivated barleys. On the basis of cDNA sequence comparisons, the GBSSI alleles can be divided into two groups representing two haplotypes. Group 1 contains GBSSI alleles from the wild-type barley cv Vogelsanger Gold and cv Oderbrucker and the amylose-free barley cv CDC Alamo. All of the members of this group lack the 15-bp insertion and vary from the group 2 alleles in 10 other positions (Table II, columns 1–4). Group 2 contains GBSSI alleles from the wild-type barley cv Shikoku Hadaka, the low-amylose lines/cultivars, Iyatomi Mochi, Waxy Oderbrucker, and SB85750, and the amylose-free barley cv Yon M Kei. All of the members of this group have the 15-bp insertion.

Comparison of GBSSI Transcripts

The relative amounts of GBSSI transcripts in developing endosperms of wild-type and *waxy* barleys were compared in two ways. First, semiquantitative RT-PCR (Fig. 4A) showed that the low-amylose line/cultivars Waxy Oderbrucker, Iyatomi Mochi, and SB85750 all had normal or only slightly reduced levels of GBSSI transcript in endosperms from grains of 30 to 45 mg fresh weight. The amylose-free barley cv Yon M Kei (containing a GBSSI allele with an intro-

duced stop codon in the coding region) also had a normal level of transcript. However, the low-amylose barley cv CDC Alamo, which had a normal amount of an inactive form of GBSSI protein, had elevated levels of transcript. Second, the transcript levels in barley cv Oderbrucker and cv Waxy Oderbrucker at two developmental stages were compared by northern analysis (Fig. 4B). In the older endosperms (from grains of 30–45 mg fresh weight), the transcripts in these cultivars were of the expected size and were of similar abundance. The GBSSI transcript was also abundant in young endosperms (from grains of 12–16 mg fresh weight) of barley cv Oderbrucker. However, in young endosperms of barley cv Waxy Oderbrucker, there was very little, if any, transcript.

Attempts to map the transcription start site for GBSSI in the low-amylose mutants and to search for alternative transcription start sites in the 5'-upstream sequences have been unsuccessful so far. We assume that there is an alternative transcription start site either in the remaining part of intron 1 or further upstream. This results in a longer pre-RNA that is spliced to give a mature RNA of similar size to the mature RNA of the wild type.
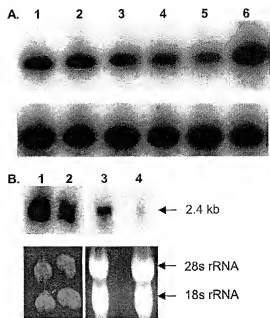
DISCUSSION

Our results suggest that there is only one GBSSI gene expressed in barley endosperm and that this is responsible for the amylose synthesized in low-amylose *waxy* cultivars as well as that in wild-type cultivars. MALDI-TOF analysis and amino acid sequence from the GBSSI protein in the endosperm of these *waxy* cultivars show that this protein is indis-

Table II. *Comparisons of the cDNA and predicted protein sequences of GBSSI from barley*

Sequences of six regions of GBSSI cDNA from different barley cultivars are compared in the six columns. The GenBank accession nos. for the cDNA sequences are given in parentheses. The positions of the bases relative to the start of translation are indicated. Bases in the cDNA sequences that vary are indicated. Amino acids in the protein sequences that vary are shown in bold. The stop codon in the Yon M Kei protein sequence is indicated by an asterisk.

Group 1	56		78	100		121	178		186	205		213	562		570	856		864
Vogelsanger Gold (X07932)	ACGGACAGA	. . . .	TTCCGGCGTCCAGGT	AAGCCGGCGATGCGGCGCTT			GGGAGCCGG			GTGGAGCGCC			CAGGAGCGC			ATTGACGGC		
	T D R	. . .	F P R R E A G	M P A . A L			G S R			V S A			Q Q R			I D G		
CDC Alamo (AF010150)	ACCGACAGA	. . . .	TTCCGGCGTCCAGGT	AAGCCGGCGATGCGGCGCTT			GGGAGCCGG			GTGGAGCGCC			CAGGAGCGC			ATTGACGGC		
	T D R	. . .	F P R R E A G	M P A . A L			G S R			V S A			Q Q R			I D G		
Oderbrucker (AF486514)	ACCGACAGA	. . . .	TTCCGGCGTCCAGGT	AAGCCGGCGATGCGGCGCTT			GGGAGCCGG			GTGGAGCGCC			CAGGAGCGC			ATTGACGGC		
	T D R	. . .	F P R R E A G	M P A . A L			G S R			V S A			Q Q R			I D G		

Group 2	56		93	115		136	193		101	220		228	577		585	871		879
Iyatomi Mochi (AF486517)	ACCGACAGACAGGTCGGCGGCGCCGCGTGGTTCCGGCATGCTGGT			AAGCCGGCGATGCGGCGGCTT			GGGAACCGG			GTGGTGCGCC			CAGTGAGCGC			ATTGACGGC		
	T D R S A P S M F R E A G			K P A D G T F			G N R			V R A			Q * R			I D G		
Waxy Oderbrucker (AF486515)	ACCGACAGACAGGTCGGCGGCGCCGCGTGGTTCCGGCATGCTGGT			AAGCCGGCGATGCGGCGGCTT			GGGAACCGG			GTGGTGCGCC			CAGTGAGCGC			ATTGACGGC		
	T D R S A P S M F R E A G			K P A D G T F			G N R			V R A			Q Q R			I D G		
SB85750 (AF486518)	ACCGACAGACAGGTCGGCGGCGCCGCGTGGTTCCGGCATGCTGGT			AAGCCGGCGATGCGGCGGCTT			GGGAACCGG			GTGGTGCGCC			CAGTGAGCGC			ATTGACGGC		
	T D R S A P S M F R E A G			K P A D G T F			G N R			V R A			Q Q R			I D G		
Shikoku Hadaka (AF486520)	ACCGACAGACAGGTCGGCGGCGCCGCGTGGTTCCGGCATGCTGGT			AAGCCGGCGATGCGGCGGCTT			GGGAACCGG			GTGGTGCGCC			CAGTGAGCGC			ATTGACGGC		
	T D R S A P S M F R H A G			K P A D G T F			G N R			V R A			Q Q R			I D G		
Yon M Kei (AF486516)	ACCGACAGACAGGTCGGCGGCGCCGCGTGGTTCCGGCATGCTGGT			AAGCCGGCGATGCGGCGGCTT			GGGAACCGG			GTGGTGCGCC			CAGTGAGCGC			ATTGACGGC		
	T D R S A P S M F R E A G			K P A D G T F			G N R			V R A			Q * R			I D G		

Figure 4. A and B. Comparison of GBSSI transcripts. A, Semiquantitative RT-PCR. RNA was extracted from endosperms of grains of 30 to 45 mg fresh weight. Tracks are: 1, barley cv Oderbrucker; 2, barley cv Waxy Oderbrucker; 3, barley cv Yon M Kei; 4, barley cv Iyatomi Mochi; 5, SB85750; and 6, barley cv CDC Alamo. Upper, Product generated using primers designed to amplify GBSSI. Lower, Product generated using primers designed to amplify Mub-1 (ubiquitin). B, Northern blots. Tracks are: 1 and 3, barley cv Oderbrucker; and 2 and 4, barley cv Waxy Oderbrucker. RNA was extracted from endosperms of grains of 30 to 45 mg fresh weight was in tracks 1 and 2 and RNA from grains of 14 to 16 mg fresh weight was in tracks 3 and 4. Upper, Products generated using primers designed to amplify GBSSI. The approximate size of the GBSSI transcript is indicated. Lower, Ethidium bromide-stained gels used to prepare the blots shown in the upper panels.

tinguishable from the GBSSI of wild-type endosperms. The protein is different from the predicted product of a second GBSSI gene, GBSSIb. GBSSIb, like the homologous gene in wheat (GBSSII, Vrinten and Nakamura, 2000), is probably expressed in parts of the plant other than the endosperm. It is likely to be responsible for the synthesis of the amylose in the pericarp, including that observed in pericarps of the amylose-free cultivars.

The GBSSI alleles of all of the low-amylose *waxy* barleys we examined carry a 413-bp deletion in the promoter and 5′-UTR. This discovery strongly suggests that the alleles in all of these cultivars are derived from a single origin. The cultivars we examined are extremely diverse in phenotype and geographical origin. They include cultivars bred in Japan, Canada, Europe, and the United States, and a wide range of awn, row, pigment, hull, and growth habit characteristics. Nonetheless, records on the origins of the cultivars are consistent with the idea that the waxy characteristic in most or all of these barleys is derived from *waxy* barleys native to Asia (Taka-

hashi, 1955). The Asian *waxy* barleys are probably all descended from a glutinous (waxy) form of barley recorded in China in the 16th century. Glutinous barleys are believed to have been introduced into Japan from Korea at some time before the 17th century (Takahashi, 1955). The European and North American *waxy* barleys stem from a U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) breeding program in Aberdeen, Idaho (1940–1950) in which a Japanese cultivar, barley cv Murasaki mochi, was crossed to a European cultivar, barley cv Oderbrucker (Harold Bockelman, USDA-ARS, personal communication). The resulting barley cv Waxy Oderbrucker was then used in a breeding program in the United States and Canada. We can trace many of the modern waxy cultivars back to barley cv Waxy Oderbrucker. For example, it was a parent of barley cv Waxy Betzes (Fox, 1981), which was then used as a parent in the breeding program in Saskatchewan from which the line SB85750 was derived. Barley cv Waxy Hector was also derived from either cv Waxy Betzes or cv Waxy Oderbrucker.

Our analysis indicates that in the low-amylose barley mutants, the 413-bp deletion alters the spatial and/or temporal expression of GBSSI in the endosperm. The deletion removes the normal transcription complex-binding site (TATA box) and transcription start site. We assume that an alternative, upstream promoter region and transcription start site are used to produce the GBSSI transcript in the mutants. The mutant transcript is produced only late in endosperm development, consistent with the appearance of amylose only later in endosperm development. The fact that amylose and GBSSI protein are found mainly or exclusively in the outer cells of the endosperm indicates that the alternative promoter specifies a different spatial and/or temporal pattern of expression from the normal promoter.

Effects on amylose content of mutations in the promoter or 5′-UTR of GBSSI have also been reported in other species. In rice, variations in amylose content between cultivars were shown to be due to differences in the efficiency with which intron 1 in the 5′-UTR was removed from the GBSSI pre-mRNA (Wang et al., 1995; Bligh et al., 1998). Low-amylose (6.7%–16.0% amylose) cultivars of rice had lower levels of mature GBSSI mRNA than high-amylose (20.0%–27.8% amylose) cultivars as well as incompletely processed GBSSI mRNA. In these cultivars, the inefficient processing of the pre-mRNA was due to a single base mutation at the 5′ splice site of intron 1. The reduced efficiency of GBSSI pre-mRNA processing also resulted in alternate splicing at multiple sites, some of which had non-consensus splice site sequences. Growth temperature can also affect the efficiency of pre-mRNA processing in low-amylose cultivars of rice. Plants grown at 18°C had higher steady-state levels of mature GBSSI mRNA than plants grown at 25 or 32°C (Larkin and Park, 1999).

At lower temperatures, when splicing was more efficient, the activity of GBSSI in the low-amylose cultivars was higher (Umemoto et al., 1995) and more amylose was synthesized (Hirano and Sano, 1998).

In potato (*Solanum tuberosum*), GBSSI activity and amylose content are affected by the presence or absence of a 140-bp fragment at a site in the promoter region approximately 0.5 kb upstream of the ATG start codon (van de Wal et al., 2001). Alleles of the gene that contain the 140-bp fragment result in lower GBSSI activity and amylose content than alleles without this fragment. The basis for this effect is not known because the variations in GBSSI activity could not be attributed to large differences in the amounts of either GBSSI RNA or protein.

The highly variable amylose content of starch granules from low-amylose cultivars of barley may be an important consideration in assessing the physicochemical properties of starch. Individual endosperms contain granules that stain blue with iodine that probably have near-normal levels of amylose, granules with blue-staining cores that are likely to have a severely reduced amylose content, and granules that stain almost completely red and probably have near-zero amylose contents. This contrasts with the situation in low-amylose starches from other species. For example, in the low-amylose lines of potato created by expression of antisense GBSSI constructs (Kuipers et al., 1994; Tatge et al., 1999), there was less granule-to-granule variation in amylose content than in the low-amylose barley cultivars. Whether the physicochemical properties of low-amylose starches of comparable bulk amylose content are influenced by the extent of heterogeneity of amylose contents between individual granules remains to be determined.

Two of the *waxy* cultivars with no amylose in the endosperm, barley cv Arizona Hulless Waxy and cv Yon M Kei, were produced by mutagenesis of wild-type cultivars rather than by breeding from low-amylose cultivars introduced to Japan (see below). Barley cv Yon M Kei was generated by mutagenesis of the wild-type barley cv Shikoku Hadaka with sodium azide (Ishikawa et al., 1995). Barley cv Arizona Hulless Waxy was generated by mutagenesis of a wild-type line, 76-19-7, with diethyl sulfate (PI 560053; USDA-ARS, National Genetic Resources Program, 1991). The third amylose-free cultivar, barley cv CDC Alamo, was derived by breeding from barley cv Arizona Hulless Waxy, and probably carries the same GBSSI allele as this parent (Bhatty and Rossnagel, 1997). Barley cv Yon M Kei and cv CDC Alamo do not have the 413-bp deletion seen in the low-amylose cultivars. Instead, they have different mutations in the GBSSI gene that account for the complete absence of GBSSI activity. The endosperm of barley cv Yon M Kei contains no detectable GBSSI protein. There is a single base substitution in this gene that creates a stop codon. This prevents the production of a full-length GBSSI protein. Presumably, the incom-

plete GBSSI protein is either unstable and is rapidly degraded or it is not capable of binding to the starch granules and, therefore, would not have been detected in our experiments. Barley cv CDC Alamo has wild-type levels of GBSSI protein. The mutant protein is able to bind tightly to starch granules like normal GBSSI, but it is unable to synthesize amylose due to a single base substitution that results in a conserved Asp residue (Asp-217) being replaced by a Val. It is known that enzymes in the glycosyltransferase family of which GBSSI is a member have Asp residues at the catalytic center that participate in the enzymatic reaction (Tarbouriech et al., 2001). Mutational analysis of several glycosyltransferases, including an isoform of starch synthase, has shown an absolute requirement for certain conserved Asp residues (for example, cellulose synthase, Saxena and Brown, 1997; chitin synthase 2, Nagahashi et al., 1995; and starch synthase IIb, Nichols et al., 2000). Substitution of these—even conservative substitution with similar amino acids—results in inactive enzymes. However, whether the conserved Asp-217 is at the catalytic center of GBSSI remains to be discovered.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Grains of barley (*Hordeum vulgare*) cultivars were obtained from the John Innes Centre and Crop Development Centre Germplasm collections, from Dr. Tom Blake (Montana State University, Bozeman, barley cv Nubet), and from Dr. Naoyuki Ishikawa (Tochigi Agricultural Experiment Station, Tochigi, Japan, barley cv Shikoku Hadaka 84 and cv Yon M Kei 286). Barley plants were grown in a greenhouse in individual pots at a minimum temperature of 12°C, with supplementary lighting in winter.

Extraction of Starch

Endosperms, free of pericarp and embryos, were dissected from developing grains of 50 mg fresh weight. For starch extraction from the inner and outer layers of the endosperm, whole endosperms were frozen on dry ice, then "peeled" with a fine razor blade to remove tissue to a depth of very approximately 0.5 mm from the surface. Starch was extracted as described by Hylton et al. (1995) and stored at −20°C.

SDS-PAGE and Immunoblotting

Starch samples were washed twice by suspension and centrifugation in 2% (w/v) aqueous SDS, at 30 mg starch mL⁻¹. Washed starch was suspended in gel sample buffer (Hylton et al., 1995) at 50 mg starch mL⁻¹, boiled for 5 min, and allowed to cool. Unlike the wild-type starches, centrifugation of the starches from the *waxy* lines did not result in a supernatant. Therefore, none of the samples were centrifuged after boiling. Instead, approximately 30 μL of the resulting paste was loaded directly into wells on 7.5% (w/v) SDS-polyacrylamide gels (10 cm long, 1 mm thick). Due to the starch in the samples, which remained in the wells, there was some unavoidable distortion of the protein bands on the gel (see Fig. 2). After electrophoresis, gels were stained with Coomassie Brilliant Blue R or electroblotted onto nitrocellulose. Blots were developed with serum containing antibodies against GBSSI of pea (*Pisum sativum*) embryos (Smith, 1990) at a dilution of 1:2,000 (v/v), as described by Denyer et al. (1997).

Identification of GBSSI Proteins by MALDI-TOF and by Sequencing

Starch granule-bound proteins were subjected to SDS-PAGE and the separated proteins were stained with Coomassie Brilliant Blue R-250. The

major, approximately 60-kD, protein bands were excised and subjected to tryptic digestion according to Speicher (2000) followed by analysis by MALDI-TOF MS. Mass fragment sizes were used to query the National Center for Biotechnology Information database using the MASCOT search tool (http://www.matrixscience.com). All matched sequences showed mass errors of <75 μL L⁻¹.

Protein was blotted from an SDS polyacrylamide gel onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA) and stained with Coomassie Brilliant Blue R250. The excised band was sequenced directly from the membrane by Edman degradation on a model 494 Procise protein sequencer (PE-Applied Biosystems, Foster City, CA) using the pulsed-liquid mode.

Light Microscopy

Freshly harvested tissue was fixed in a formaldehyde solution and dehydrated through a graded ethanol series according to Johnson et al. (1994). After transfer to Histoclear (Agar Scientific, Stansted, Essex, UK), tissue was embedded in Paramat wax (BDH, Poole, UK), and sectioned. Wax was removed with Histoclear and sections were transferred through an ethanol series into water and photographed through a light microscope after staining with iodine solution (dilutions of 2- or 5-fold of Lugol's solution, Sigma, Poole, UK). To improve contrast, red/brown and blue colors in photographs were enhanced using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

DNA Extraction and the Cloning and Sequencing of Alleles of GBSSI

DNA was extracted from 0.1-g samples of young barley leaves with the DNeasy plant mini-kit (Qiagen, Hilden, Germany). The promoters were amplified using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) with primers designed to GBSSI from barley cv Vogelsanger Gold (X07931) forward (5'-TATATGACGCACTCCACACCCACACACACA-3') and reverse (5'-CTGTTCCTGAAATCTAAGATCGTTTGCAGA-3'). The bluntended PCR products had adenine residues added by incubation with deoxyadenylate triphosphate and *Taq* polymerase at 72°C. The products were then ligated into pGEM-T-easy vector (Promega, Madison, WI) for sequencing. Overlapping sequencing primers were designed at 400-bp intervals.

RNA Extraction and DNA Synthesis

RNA was extracted from 0.5 g of endosperms from grains of 30 to 45 mg each or from 21 whole grains of 12 to 16 mg each using Concert RNA reagent (Invitrogen Ltd., Paisley, UK). RNA was treated with DNaseI (Roche, Basel) and cleaned again with phenol:chloroform. cDNA was synthesized at 58°C from a reverse primer designed to Vogelsager Gold (X07932; P2, 5'-TGCTCCATGCACCAGAATGT-3') using Thermoscript RT (Invitrogen Ltd.). The enzyme was denatured at 85°C and RNA removed from the duplex by incubation at 37°C with RNase H (Roche).

RT-PCR

RT-PCR with the cDNA synthesis primer (P2) and a forward primer (P1, 5'-TGCTCTCTCACTGCAGGTAG-3') was done using Pfx polymerase or Platinum *Taq* DNA polymerase and PCR, reaction buffer (Invitrogen Ltd.). Semiquantitative RT-PCR was done in tandem with primers to ubiquitin (mub1-M60175; 5'-CGGACACCATCGACAACGTCCAG-3' and 5'-GCCA-GTTCTAAGCCTTCTGGTTGTTAG-3'). PCR cycles were paused after 15 cycles and 5-μL aliquots removed. Products were separated on 1% (w/v) agarose gel and transferred to Duralon nylon membrane (Stratagene) by capillary transfer. Membranes were hybridized in phosphate buffer (pH 7.4), 10 mM EDTA, and 7% (w/v) SDS with 25 ng of 32P[α-dCTP]-labeled GBSSI-cDNA or ubiquitin-cDNA amplified from Oderbrucker, for 4 h at 65°C and washed with 0.1× SSC containing 0.1% (w/v) SDS.

Cloning and Sequencing of GBSSIb

RNA was extracted from the entire seeds of barley cv Nubet 3 DPA. Ten micrograms of total RNA was used to synthesize cDNA using the Generacer

Kit (Invitrogen Ltd.). RACE products amplified with primers designed to EST AL508718 (5′-TCCTACAACTGGAACAGACTTCCGAGATAA-3′ and 5′-ACGGTTCTGCTTTTGTGCTTGCTGCATT-3′) were cloned into the TOPO-10 vector (Invitrogen Ltd.) and sequenced.

Northern Blotting

Ten micrograms of total RNA was separated on a 1% (w/v) agarose denaturing gel using ethidium bromide under UV light to ensure equal loading. RNA was transferred to Duralon nylon membrane (Stratagene) by capillary transfer. Hybridization conditions were identical to those used for semiquantitative RT-PCR.

LITERATURE CITED

Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the *waxy* protein (granule-bound starch synthase) in wheat. Plant Mol Biol 22: 67–82

Andersson L, Fredriksson H, Bergh MO, Andersson R, Åman P (1999) Characterisation of starch from inner and peripheral parts of normal and waxy barley kernels. J Cereal Sci 30: 165–171

Banks W, Greenwood CT, Walker JT (1970) Studies on the starches of barley genotypes: the waxy starch. Stärke/Starch 22: 149–180

Bhatty RS, Rossnagel BG (1997) Zero amylose lines of hulless barley. Cereal Chem 74: 190–191

Bligh HFJ, Larkin PD, Roach PS, Jones CA, Fu H, Park WD (1998) Use of alternate splice sites in granule-bound starch synthase mRNA from low-amylose rice varieties. Plant Mol Biol 38: 407–415

Denyer K, Barber LM, Edwards EA, Smith AM, Wang TL (1997) Two isoforms of the GBSS class of granule-bound starch synthase are differentially expressed in the pea plant. Plant Cell Environ 20: 1566–1572

Drescher A, Schreiber H, Habekuss A (2000) Mutations at the waxy locus of different barleys and their influence on starch synthesis. Barley Genetics VIII 3: 142–144

Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300: 1005–1016

Fox GJ (1981) The effect of the waxy endosperm gene, short awn, and hulless seed genes upon biochemical and physiological seed characteristics important in barley (*Hordeum vulgare* L.). PhD thesis. Montana State University, Bozeman

Fujita N, Taira T (1998) A 56-kD protein is a novel granule-bound starch synthase existing in the pericarps, aleurone layers, and embryos of immature seed in diploid wheat (*Triticum monococcum* L.). Planta 207: 125–132

Hirano H-Y, Sano Y (1998) Enhancement of *Wx* gene expression and the accumulation of amylose in rice from rupture to cold temperatures during seed development in rice. Plant Cell Physiol 39: 807–812

Hylton CM, Denyer K, Keeling PL, Chang M-T, Smith AM (1995) The effect of *waxy* mutations on the granule-bound starch synthases of barley and maize endosperms. Planta 198: 230–237

Ishikawa N, Ishihara J, Itoh M (1995) Artificial induction and characterization of amylose-free mutants of barley. Barley Genet Newslett 24: 49–53

Johnson S, Liu CM, Hedley CL, Wang TL (1994) An analysis of seed development in *Pisum sativum*: XVIII. The isolation of mutants defective in embryo development. J Exp Bot 45: 1503–1511

Kuipers AGJ, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato tuber are affected by the reduction of granule-bound starch synthase gene expression. Plant Cell 6: 43–52

Larkin PD, Park WD (1999) Transcript accumulation and utilization of alternate and non-consensus splice sites in rice granule-bound starch synthases are temperature-sensitive and controlled by a single-nucleotide polymorphism. Plant Mol Biol 40: 719–727

McDonald AML, Stark JR, Morrison WR, Ellis RP (1991) The composition of starch granules from developing barley genotypes. J Cereal Sci 13: 93–112

Morrison WR, Scott DC, Karkalas J (1986) Variation in the composition and physical properties of barley starches. Stärke/Starch 38: 374–379

Nagahashi S, Sudoh M, Ono N, Sawada R, Yamaguchi E, Uchida Y, Mio T, Takagi M, Arisawa M, Yamada-Okabe H (1995) Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. Implication of two highly conserved domains as possible catalytic sites. J Biol Chem 270: 13961–13967

Nakamura T, Vrinten P, Hayakawa K, Ikeda J (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol 118: 451–459

Nichols DJ, Keeling PL, Spalding M, Guan H (2000) Involvement of conserved aspartate and glutamate residues in the catalysis and substrate binding of maize starch synthase. Biochem 39: 7820–7825

Olsen O-A, Linnestad C, Nichols SE (1999) Developmental biology of the cereal endosperm. Trends Plant Sci 4: 253–257

Oscarsson M, Parkkonen T, Autio K, Åman P (1997) Composition and microstructure of waxy, normal and high amylose barley samples. J Cereal Sci 26: 259–264

Rohde W, Becker D, Salamini F (1988) Structural analysis of the waxy locus from *Hordeum vulgare*. Nucleic Acids Res 16: 7185–7186

Saxena IM, Brown RM (1997) Identification of cellulose synthase(s) in higher plants: sequence analysis of processive β-glycosyltransferases with the common motif "D,D,D35Q(Q,R)XRW." Cellulose 4: 33–49

Smith AM (1990) Evidence that the "waxy" protein of pea is not the major starch-granule-bound starch synthase. Planta 182: 599–604

Song Y, Jane J (2000) Characterization of barley starches of waxy, normal and high amylose varieties. Carbohydr Polymers 41: 365–377

Speicher KD (2000) Systematic analysis of peptide recoveries from in-gel digestions for protein identifications in proteome studies. J Biomol Technol 11: 74–86

Taira T, Fujita N, Takaoka K, Uematsu M, Wadano A, Kozaki S, Okabe S (1995) Variation in the primary structure of waxy proteins (granule-bound starch synthase) in diploid cereals. Biochem Genetics 33: 269–281

Takahashi R (1955) The origin and evolution of cultivated barley. Adv Genetics 7: 227–266

Tarbouriech N, Charnock SJ, Davies GJ (2001) Three-dimensional structures of the Mn and MgdTDP complexes of the family GT-2 glycosyltransferase SpeA: a comparison with related NDP-sugar glycosyltransferases. J Mol Biol 314: 655–661

Tatge H, Marshall J, Martin C, Edwards EA, Smith AM (1999) Evidence that amylose synthesis occurs within the matrix of the starch granule in potato tubers. Plant Cell Environ 22: 543–550

Umemoto T, Nakamura Y, Ishikura N (1995) Activity of starch synthase and the amylose content in rice endosperm. Phytochemistry 40: 1613–1616

USDA-ARS, National Genetic Resources Program (1991) Pl 560053. Germplasm Resources Information Network (GRIN)-Online database www.ars-grin.gov. National Germplasm Resources Laboratory, Beltsville, MD

van de Wal MHBJ, Jacobsen E, Visser RGF (2001) Multiple allelism as a control mechanism in metabolic pathways: GBSSI allelic composition affects the activity of granule-bound starch synthase I and starch composition in potato. Mol Gen Genomics 265: 1011–1021

Vrinten P, Nakamura T (2000) Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol 122: 255–263

Wang Z-Y, Zheng F-Q, Shen G-Z, Goa J-P, Snustad DP, Li M-G, Zhang J-L, Hong M-M (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the *waxy* gene. Plant J 7: 613–622

Discrete Forms of Amylose Are Synthesized by Isoforms of GBSSI in Pea[W]

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Amyloses with distinct molecular masses are found in the starch of pea embryos compared with the starch of pea leaves. In pea embryos, a granule-bound starch synthase protein (GBSSIa) is required for the synthesis of a significant portion of the amylose. However, this protein seems to be insignificant in the synthesis of amylose in pea leaves. cDNA clones encoding a second isoform of GBSSI, GBSSIb, have been isolated from pea leaves. Comparison of GBSSIa and GBSSIb activities shows them to have distinct properties. These differences have been confirmed by the expression of GBSSIa and GBSSIb in the amylose-free mutant of potato. GBSSIa and GBSSIb make distinct forms of amylose that differ in their molecular mass. These differences in product specificity, coupled with differences in the tissues in which GBSSIa and GBSSIb are most active, explain the distinct forms of amylose found in different tissues of pea. The shorter form of amylose formed by GBSSIa confers less susceptibility to the retrogradation of starch pastes than the amylose formed by GBSSIb. The product specificity of GBSSIa could provide beneficial attributes to starches for food and nonfood uses.

INTRODUCTION

Starch is composed of two glucan polymers: amylose, a predominantly linear α-1,4-linked glucan, and amylopectin, in which the α-1,4-linked chains are branched extensively by 1,6-linkages. In higher plants, starch is made both in chloroplasts ("transitory" starch) in leaves and in amyloplasts ("reserve" or "storage" starch) in nonphotosynthetic storage organs. The small, disc-shaped granules of transitory starch show little variation between different plant species (French, 1984). By contrast, the size and shape of storage starch, which forms relatively long-term reserves and is designed to be compact and stable but is metabolized readily on demand, is extremely diverse and species specific (for review, see Wang et al., 1998).

Both the ratio of amylose to amylopectin in storage starch and the average size of the two polymers show significant variation between different plant species. These parameters also may differ between transitory and storage starch within

one species and can vary with the developmental age of the plant organ and even within the granules themselves (Jane and Shen, 1992; Yun and Matheson, 1992). These parameters are important in determining the physical characteristics of starch, such as swelling, solubility, pasting, viscosity, and retrogradation.

In storage starch, amylose content can vary between 11 and 37%, but on average, it constitutes 30% of the total glucan (Shannon and Garwood, 1984). In transitory starch, its contribution is generally lower. The starch from rice and pea leaves contains <15% amylose (Taira et al., 1991; Tomlinson et al., 1997), and the starch of young tobacco leaves contains 15 to 17% amylose (Matheson and Wheatley, 1962).

The molecular mass of amylose varies between species. For example, wheat amylose has an average degree of polymerization of 570 Glc units (equivalent to a molecular mass of ~100 kD) compared with potato amylose, which is reported to have an average degree of polymerization of between 250 kD and 1 MD) (Hizukuri and Takagi, 1984; Jane and Shen, 1992). It does contain some branches, but the frequency and length of these branches differ between the storage starches of different species (Shannon and Garwood, 1984).

Both amylose and amylopectin are synthesized by starch synthases, which catalyze the transfer of Glc from ADP-Glc

to the nonreducing end of glucan chains via an α-1,4-linkage. One isoform of starch synthase is bound exclusively to starch granules (granule-bound starch synthase I [GBSSI]). The other isoforms of starch synthase, which are both located in the plastid stroma and to varying extents bound to starch granules, synthesize amylopectin (soluble starch synthases). There is considerable evidence from mutation and antisense studies to suggest that specific starch synthase isoforms play different qualitative roles in determining amylopectin branch length distribution (Craig et al., 1998; Edwards et al., 1999b; Lloyd et al., 1999). However, the soluble starch synthases do not appear to be involved in amylose synthesis.

GBSSI is bound tightly to the starch granule. The sequence of GBSSI is known for many plant species (Mason-Garner et al., 1998). In contrast to the more diverse soluble isoforms, it is a very highly conserved protein of ~60 kD. Amylose synthesis is associated with GBSSI activity. The *waxy* mutants of cereals (Weatherwax, 1922; Murata et al., 1965; Ishikawa et al., 1994; Nakamura et al., 1995), the *amf* mutant of potato (Hovenkamp-Hermelink et al., 1987), the *lam* mutants of pea (Denyer et al., 1995), and GBSSI antisense lines of potato (Visser et al., 1991) all involve the reduction or elimination of GBSSI activity and a specific reduction or elimination of amylose in the starch of the storage organs of these species.

The mechanisms by which different isoforms of starch synthase catalyze the same reaction and yet generate polymer variation are not fully understood, but there is increasing evidence to suggest that isoform localization within the plastid and fundamental differences in kinetics and reaction mechanisms are important determinants of product specificity (Smith et al., 1997; Denyer et al., 1999; Edwards et al., 1999a; Imparl-Radosevich et al., 1999; Commuri and Keeling, 2001). Furthermore, recent work has suggested that GBSSI, like the soluble starch synthases, may exist as more than one isoform.

The leaves and stem of *waxy* rice, the leaves and pericarp of *waxy* maize, the pericarp of *waxy* wheat, and the pods, leaves, and nodules of *lam* pea all contain amylose, implying that another gene (or genes) controls amylose production in the organs of these plants (Shannon and Garwood, 1984; Taira et al., 1991; Denyer et al., 1997; Nakamura et al., 1998). A protein of similar molecular mass, antigenic properties, and N-terminal sequence to GBSSI has been detected in the leaves of *lam* pea (Denyer et al., 1997), and a cDNA encoding a GBSSI-like starch synthase has been isolated from *waxy* wheat and shown to be expressed in nonstorage tissues (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000).

The existence of two different forms of GBSSI, expressed in different parts of the plant in which the starches have a different polymer composition, suggests that GBSSI isoforms might have distinct characteristics that make specific contributions to amylose synthesis, although the structural similarities between GBSSI proteins are so high that this

is not an obvious inference. In potato, by contrast, only one isoform of GBSSI has been described, and mutation of the gene encoding this protein (*AMF*) results in the complete elimination of amylose from tubers, leaves, and roots (Hovenkamp-Hermelink et al., 1987). This finding suggests that there is only one isoform of GBSSI active in the major starch-synthesizing tissues of potato.

In this article, we compare the molecular mass of amylose partially purified from the starch of pea leaves and embryos by gel-permeation chromatography and show that pea leaves contain an amylose that is distinct in its average molecular mass from that in embryos. A cDNA clone encoding a second isoform of pea GBSSI, designated GBSSIb (Denyer et al., 1997), is expressed in the leaves of pea and at a low level in pea embryos, where the first isoform, GBSSIa, is very highly expressed (Dry et al., 1992). Characterization of the in vitro activities of the two isoforms after the expression of both in *Escherichia coli* shows them to have distinct properties.

We analyzed the nature of their products in vivo by expression of each in an amylose-free (*amf*; a GBSSI mutant) potato and show that both produce amylose but that their products are distinct. The molecular mass of amylose synthesized by GBSSIb in *amf* potato was the same as that of pea leaf amylose, but the amylose synthesized by GBSSIa was smaller. A difference in the physical properties of the starches synthesized by GBSSIa and GBSSIb also was observed. The significance of this difference with respect to the functional behavior of the polymers is discussed.

RESULTS

Pea Leaf Amylose Has a Greater Molecular Mass Than Pea Embryo Amylose

Amylose was partially purified from pea leaf and pea embryo starch by butanol precipitation, and its molecular mass distribution was analyzed by gel filtration chromatography. The results are shown in Figure 1. For both leaf and embryo, an initial contaminating amylopectin-containing peak (wavelength of maximal absorbance [λ_{max}] of 550 nm) was eluted in fractions 18 to 23. Pea leaf amylose (λ_{max} of 610 nm) appeared to have a higher molecular mass (peak elution fractions 38 to 41, corresponding to a dextran equivalent peak molecular mass of 655,390 ± 10 D) (Figure 1A) than pea embryo amylose (λ_{max} of 620 nm; peak elution fractions 41 and 42, corresponding to a dextran equivalent peak molecular mass of 470,210 ± 10 D) (Figure 1B). The high λ_{max} values indicate that the amylose fractions had little or no contamination with branched glucans.

A cDNA encoding the major GBSSI protein in pea embryos, GBSS1a, has been cloned and characterized (Dry et
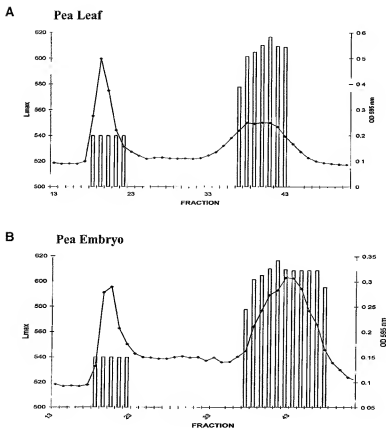
Figure 1. Gel Permeation Chromatography of Partially Purified Amylose from Embryos and Leaves of Pea.

Amylose was partially purified by butanol precipitation, solubilized, and subjected to chromatography on Sepharose CL-2B columns. Fractions eluted from the columns were mixed with an iodine solution, and absorbance was measured at 595 nm (closed circles). The wavelength of maximum absorbance of the starch-iodine complex (λ_{max}) also was determined (bars).
(A) Amylose from pea leaves.
(B) Amylose from pea embryos.
Results from typical experiments are shown. Lmax represents the λ_{max} scale in nm.

al., 1992). To determine whether the difference in molecular mass of the amyloses in leaf and embryo could be attributed to the activity of different GBSSI isoforms, we isolated a cDNA clone encoding a GBSSI that was highly expressed in pea leaves (GBSSIb) and compared its properties in vitro and in vivo with those of GBSSIa.

Isolation and Characterization of a Full-Length cDNA Clone for Pea GBSSIb

cDNA clones encoding the GBSSI isoform expressed highly in pea leaves were identified from a cDNA library made to mRNA from pea leaves using the potato GBSSI cDNA as a

probe and washing at low stringency. The three positive clones isolated were identical over regions of shared sequence. The longest GBSSIb cDNA was 2100 bp in length, which included a 68-bp poly(A)$^+$ tail and an open reading frame of 1839 bp flanked by 5' and 3' untranslated regions of 5 and 87 bp, respectively.

The derived amino acid sequence of GBSSIb predicts a 613–amino acid polypeptide of 67.6 kD, which shows significant similarity (68.8% identity and 75.6% similarity) to the predicted sequence of pea GBSSIa (Figure 2A). The N-terminal region of full-length GBSSIb contains the amino acid sequence 5'-GMNLIFVGTEVAPWSKTGGLGDVL-3', which is identical to that obtained from protein sequencing of GBSSIb extracted from *lam* pod starch (Denyer et al., 1997).

Figure 2. Comparative Analysis of Pea GBSSI Isoforms.

(A) Comparison of the derived amino acid sequences of pea GBSSIb (top) and pea GBSSIa (bottom) aligned by GAP (gap weight of 3.0, length weight of 0.1; Devereux et al., 1984). The first amino acid of the mature GBSSIa and GBSSIb proteins is boxed in dark gray. The N-terminal KTGGL motif thought to interact with ADP/ADP-Glc and the C-terminal KTGGL look-alike motif thought to influence kinetic properties are boxed in light gray.

This sequence corresponds to the N terminus of mature GBSSIb, which is predicted to have an 85–amino acid transit peptide.

Mature GBSSIb is a protein of 58.4 kD (528 amino acids) and has a predicted pI of 6.44. Mature GBSSIa is a protein of 58.3 kD (528 amino acids) with a predicted pI of 6.22. The two mature peptide sequences are 73% identical (80% similar). Both possess the N-terminal KTGGL motif thought to be responsible for binding ADP/ADP-Glc and the C-terminal KTGGL "look-alike" motif that has been shown to influence the kinetic properties of potato GBSSI in vitro (Edwards et al., 1999a).

However, a database search showed that mature GBSSIb is more similar (76.5% identity and 83.5% similarity) to mature potato GBSSI than it is to GBSSIa from pea. A phylogenetic tree of GBSSI from several species is shown in Figure 2B. There appear to be two major classes. One class consists entirely of GBSSI forms from dicots and one isoform from wheat, GBSSII, which is expressed in leaves (Vrinten and Nakamura, 2000). This second class is subdivided, with the pea embryo GBSSIa clustering with the GBSSI from another legume, bean.

Differential Expression of GBSSI Isoforms in Pea

The expression of GBSSIa and GBSSIb in pea leaf and pea embryo was examined by quantitative reverse transcriptase–mediated PCR, which indicated that the two isoforms were expressed differentially. GBSSIa was found to be highly expressed in embryos. The GBSSIa transcript also was detected in leaves. These results confirmed data previously reported by Dry et al. (1992). Low levels of the GBSSIb transcript were detected in embryo. GBSSIb was most highly expressed in leaves, particularly young leaves (Figure 3).

Expression of GBSSIa and GBSSIb in E. coli

To determine whether the different isoforms of GBSSI from pea had different enzyme specificities, both proteins were expressed in E. coli for analysis of their kinetic properties. For all expression analyses, GBSSI proteins with an N-terminal S-tag fusion peptide were used. Both GBSSIa and
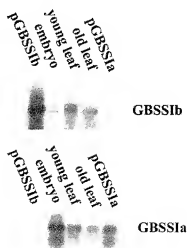
GBSSIb

GBSSIa

Figure 3. Expression of GBSSIa and GBSSIb in Leaves and Embryos of Pea.

DNA gel blots of cDNA amplified by PCR. cDNA was made to poly(A)⁺ RNA from pea embryos, young leaves, and fully expanded (old) leaves and probed with either a GBSSIa-specific probe or a GBSSIb-specific probe. Control lanes contained fragments amplified from plasmids containing GBSSIa cDNA (pGBSSIa) and GBSSIb cDNA (pGBSSIb).

GBSSIb were synthesized in approximately the same amounts in the soluble phase of E. coli (0.024 to 0.026 µg/µL, as measured by the S-tag assay), and both had the correct molecular mass of ~60 kD, as judged by protein gel blot analysis (Figure 4).

No significant starch synthase activity was found associated with GBSSIa under any of the conditions tested. This agrees with the results obtained when GBSSIa was solubilized from pea embryos (Smith, 1990) and suggests that this isoform is inactive when not bound to starch granules and that the appropriate conditions for assay in solution have not yet been established.

In contrast, GBSSIb had activity that was very similar to that of GBSSI from potato tuber (Edwards et al., 1999a). The

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Figure 2. (continued).

(B) Relationships between GBSSI proteins of different species. An unrooted phylogenetic tree was assembled using the Phylogeny Interference Package, version 3.5. A maximum likelihood tree was calculated using PUZZLE, and the tree was drawn using NEIGHBOR (Stimmer and von Haeseler, 1996, 1997). The distances along the branch lengths are proportional to the similarity between the sequences as calculated using an unweighted pair-group method based on arithmetic averages specified in the PILEUP program (Devereux et al., 1984). The GBSSI enzymes fall into two distinct families.
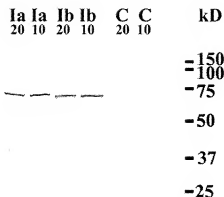
Ia Ia Ib Ib   C C   kD
20 10 20 10   20 10

=150
=100
—75
—50
—37
—25

Figure 4. Expression of Pea GBSSIa and GBSSIb S-Tag Fusion Proteins in *E. coli*.

Protein gel blot of a SDS–polyacrylamide gel of soluble extracts of *E. coli* cells developed with S protein–alkaline phosphatase conjugate. Each pair of lanes contained 10 or 20 μg of protein as indicated. Lanes 1 and 2, colonies transformed with pStag19 (expressing GBSSIa); lanes 3 and 4, colonies transformed with pStag46 (expressing GBSSIb); lanes 5 and 6, colonies transformed with vector alone (pStag); lane 7, S-tagged marker proteins. Molecular mass markers are shown at right in kD.

kinetics of pea GBSSIb and potato GBSSI (both expressed in *E. coli*) are compared in Tables 1 and 2. GBSSIb is a more active enzyme than potato GBSSI. Its specific activity of 0.6 μmol ADP-Glc·min⁻¹·mg⁻¹ at 5 mg/mL amylopectin and 1 mM ADP-Glc was somewhat higher than that of the potato enzyme (0.2 μmol ADP-Glc·min⁻¹·mg⁻¹) under the same conditions, as was the activation by 50 mg/mL amylopectin and by 0.5 M citrate (Table 2). The specific activity of GBSSIb with maltooligosaccharides was approximately threefold greater than that of potato, although the activa-

tion in combination with amylopectin was approximately the same.

Like potato GBSSI, pea GBSSIb exhibited a lag in initiating glucan synthesis of 1 to 4 min, depending on amylopectin concentration (data not shown). GBSSIb showed processive rather than distributive activity (the enzyme preferentially added successive Glc units to a single glucan chain rather than to different chains) when supplied with 100 mM malto-triose and 2.5 mg/mL amylopectin (data not shown), as does potato GBSSI (Denyer et al., 1999; Edwards et al., 1999a).

Expression of GBSSIa and GBSSIb in the *amf* Mutant of Potato

We were unable to detect any activity from solubilized GB-SSIa from pea embryos or from GBSSIa expressed in *E. coli* and assayed in vitro. To determine whether the enzyme encoded by this cDNA was capable of synthesizing amylose, we introduced it under the control of a strong promoter into the *amf* mutant of potato, which does not make amylose (as a result of mutation of the GBSSI gene; Hovenkamp-Hermelink et al., 1987). We compared the activity of GBSSIa with that of GBSSIb from pea controlled by the same promoter in *amf*. Further controls included the *amf* mutant, a wild-type diploid line (which was formed by doubling up from the haploid progenitor of the *amf* line (Visser et al., 1989]; the *amf* line also is diploid after chromosome doubling of the haploid), and the *amf* mutant transformed with the genomic clone of potato GBSSI (BW101; van der Leij et al., 1991). Starch from developing tubers of independent transformants was screened for the presence of GBSSI by Coomassie blue staining of polyacrylamide gels and by protein gel blot analysis (Figure 5). For the GBSSIa lines, 24 of 50 transformants were found to express substantial amounts of GBSSIa comparable to the amount of GBSSI found in wild-type potato (Figure 5A). Lines Ia.5, Ia.7, Ia.12, and Ia.3 (used as a control because no GBSSI protein was visible) were

Table 1. Comparison of the Activities of GBSSIb from Pea and GBSSI from Potato Tuber

Enzyme	AP (mg/mL)	ADP-Glc (mM)	AP K_m (mg/mL)	ADP/Glc K_m (mM)	V_{max} (μmol·min⁻¹·mg⁻¹)
Pea leaf GBSSIb	0–25	1	15.0 ± 7.0		2.7 ± 1.0
	5	0–2		0.9 ± 0.1	1.2 ± 0.2
	50	0–2		1.3 ± 0.2	19 ± 4.0
Potato GBSSI	0–25	1	10.0 ± 1.0		0.6 ± 0.0
	5	0–2		1.3 ± 0.1	0.5 ± 0.0
	50	0–2		1.1 ± 0.2	2.0 ± 0.0

cDNA clones encoding each protein were expressed in *E. coli* with an N-terminal S-tag attached to the mature protein (i.e., without the transit peptide). To establish the kinetic responses of the enzymes, assays were performed at different amylopectin and ADP-glc concentrations. AP, amylopectin.

Table 2. Activity of Pea GBSSIb and Potato GBSSI in the Presence of Different Concentrations of Amylopectin Substrate and Other Effectors

Substrates	Pea Leaf GBSSIb Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ GBSSI protein)	Potato GBSSI Activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$ GBSSI protein)
5 mg/mL amylopectin	0.6 ± 0.1	0.2 ± 0.0
50 mg/mL amylopectin	2.9 ± 1.5	1.1 ± 0.2
Fold activation by amylopectin	7.3	5.6
0.5 M citrate + 5 mg/mL amylopectin	6.4 ± 3.2	1.8 ± 0.6
Fold activation by citrate	10.7	9.3
0.5 M citrate + 50 mg/mL amylopectin	17.6 ± 8.8	2.7 ± 0.6
Fold activation by citrate at 50 mg/mL amylopectin	6.0	2.5
Fold activation by amylopectin at 0.5 M citrate	2.8	1.5
50 mM MOS	0.3 ± 0.1	0.1 ± 0.0
Fold activity relative to 5 mg/mL amylopectin	0.5	0.5
100 mM MOS + 5 mg/mL amylopectin	2.4 ± 0.1	0.6 ± 0.0
Fold activation by amylopectin on MOS	8	6

MOS, maltooligosaccharides.

used for further analysis. The transformation efficiency of plasmid pBinGBSSIb was approximately the same as that of pBinGBSSIb, but the expression levels of GBSSIb generally were relatively low, especially compared with the amount of GBSSI in wild-type potato (Figure 5B).

This may have been because the gene construct had a rather short leader sequence (49 nucleotides), which could have limited the efficient translation of GBSSIb in transgenic potato, or because unusual codons at the start of the GBSSIb sequence limited translation in potato because the levels of GBSSIa and GBSSIb transcript were equivalent in the transgenic lines used for further analysis (data not shown). The GBSSIb-expressing lines Ib.12 and Ib.4 were studied further, together with the nonexpressing line Ib.8.

Starch Synthase Activity and Protein Synthesis in *amf* Potato

The activities of granule-bound starch synthase from the transgenic lines are summarized in Table 3. These activities were assayed on the isolated and washed starch granules and did not involve solubilization of GBSSI. All of the lines expressing GBSSI protein exhibited significantly higher starch synthase activity on the granules than the *amf* control. When the transgenic plants were corrected for background activity measured in the *amf* control, Ia.12, with the highest level of GBSSIa expression, had activity that was between 14.5 and 23.7% of that of the wild type or the fully complemented mutant (BW101), whereas Ib.12, with the highest level of GBSSIb expression, had activity between 5.7 and 9.3% of that of the wild type or the fully complemented mutant (BW101).

When these values were compared with the amounts of GBSSI made in each line (Table 4), it was clear that GBSSIa encoded an active GBSSI. However, the specific activity of

GBSSIb in the *amf* background was much higher than that of GBSSIa. Ia.12 produced GBSSIa protein at a level equivalent to the level of potato GBSSI in wild-type potato (0.25 μg GBSSI protein/mg starch; Figure 5A), whereas Ib.12 synthesized 250 times less GBSSIb protein (0.001 μg GBSSI protein/mg starch; Figure 5B). The results are summarized in Table 4 and show that although GBSSIa is active in vivo, GBSSIb encodes a distinct protein with a significantly higher specific activity than GBSSIa (at least 10-fold higher) in vivo.

Interestingly, comparison of enzyme activities in solution (Table 1) and on the starch granule (Table 4) implies that both proteins are activated significantly by association with the starch granule. In fact, activation probably requires the incorporation of GBSSIa into the starch granule, because in vitro assays of soluble GBSSIa (expressed in *E. coli*) with added starch granules did not show starch synthase activity. The precise degree of activation was impossible to calculate for GBSSIa, because no activity could be detected for the soluble form. For GBSSIb, the specific activity of the soluble form expressed in *E. coli* was calculated to be 0.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ GBSSI protein (with maltooligosaccharides as substrate; Table 2), and on the *amf* starch, it was measured as 3.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ GBSSI protein (a 10-fold activation).

High-Performance Anion-Exchange Chromatography Analysis of the Soluble Products of GBSSIa and GBSSIb

To determine whether GBSSIa and GBSSIb expressed in the transgenic *amf* lines have a processive or a distributive reaction mechanism, isolated starch granules from these lines were incubated with ADP-^{14}C-Glc, maltotriose, and amylopectin, and the sizes of the reaction products were analyzed by high-performance anion-exchange chromatography. Control granules from the *amf* line and the *amf* line
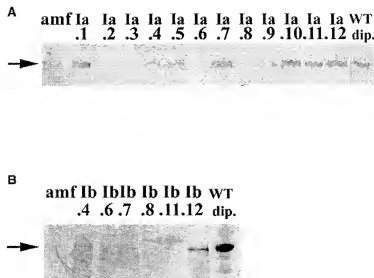
Figure 5. Expression of Pea GBSSIa and GBSSIb in *amf* Potato Tubers.

(A) Immunoblots of purified starch from lines transformed with pea GBSSIa.
(B) Immunoblots of purified starch from lines transformed with pea GBSSIb.
Blots were developed with antiserum to pea GBSSIa at a dilution of 1:1000 (Smith, 1990). GBSSIa lines Ia.5, Ia.7, Ia.12, and nonexpressing line Ia.3 and GBSSIb lines Ib.12, Ib.4, and nonexpressing line Ib.8 were used for further analysis. GBSSI on wild-type diploid starch (WT dip.) is shown for comparison with GBSSIa and GBSSIb protein levels. Arrows indicate the positions of the GBSSIa and GBSSIb proteins.

expressing potato GBSSI (BW101) were used. The *amf* granules made only maltotetrose (as a result of the distributive reaction of endogenous potato SSII and SSIII). BW101 granules (expressing potato GBSSI) displayed a highly processive reaction. Granules expressing either pea GBSSIa or GBSSIb showed processive elongation of glucan chains (data not shown).

Analysis of Transgenic Starch

Iodine Staining

Transgenic starch was stained with Lugol's iodine and compared with the *amf* line stained red-brown because of the absence of amylose (Figure 6B). Wild-type starch granules stained blue with clearly defined darker staining rings caused by the presence of amylose (Figure 6A). Blue-staining material was seen in both Ia and Ib lines expressing the pea GBSSI proteins (Figures 6C and 6D). In the Ia lines, the blue-staining material was confined essentially to a series of rings, although sometimes there were granules with solid blue-staining cores. The rings tended to fade toward the periphery, but the staining pattern was quite uneven.

In some granules, the most intensely staining ring was closer to the periphery than to the center. In the Ib lines, there were many fewer blue cores and fewer but more evenly stained rings. These differences in amylose localization were reproduced in independent transgenic lines. The significance of these differences in staining pattern between GBSSIa and GBSSIb starch is under investigation, but they suggest that the two isoforms may synthesize amylose preferentially in different regions of the starch granule.

Table 3. Starch Synthase Activity Measured on Starch Granules from Potato Lines

Line	GBSS Activity (nmol·min^{-1}·mg^{-1} starch)
Amf	0.008 ± 0.001
Diploid	0.287 ± 0.061
BW101	0.463 ± 0.061
Peala.3	0.018 ± 0.004
Peala.5	0.048 ± 0.011
Peala.7	0.067 ± 0.009
Peala.12	0.074 ± 0.001
Pealb.8	0.009 ± 0.003
Pealb.4	0.032 ± 0.010
Pealb.12	0.034 ± 0.006

Quantitative and Qualitative Analysis of Amylose

Amylose, as judged by iodine staining, was synthesized by both GBSSIa and GBSSIb in the *amf* background. The amylose contents of the starch from the highest expressing lines for Ia.12 and Ib.12 were calculated to be 1 and 0.8%, respectively, compared with 20% for the wild-type diploid and the potato GBSSI-complemented *amf* line, BW101. This represents a 5% restoration of amylose synthesis by pea GBSSIa and a 4% restoration by pea GBSSIb.

In the case of pea GBSSIb, the failure of the pea protein to fully complement amylose production in the *amf* line probably was attributable to the low levels of GBSSIb production achieved (Figure 5B). However, in the case of GBSSIa, high levels of protein (equivalent to those of wild-type GBSSI) were produced in the transgenic lines (Figure 5A). This finding implies that GBSSIa encodes an isoform of GBSSI distinct from that in potato that cannot fully complement potato GBSSI activity.

When amyloses from lines Ia.12 and Ib.12 were concentrated by butanol precipitation and analyzed by gel permeation chromatography, they had different molecular masses (Figures 7A and 7B). In line Ia.12, the mass of amylose (peak fractions 42 to 43; peak measured as 367,320 ± 10 D) was significantly smaller than that of Ib.12 (peak fraction 39; peak measured as 655,390 ± 10 D). Amylose from line Ib.12 appeared to have a molecular mass that was very similar to that of pea leaf amylose (peak at 655,390 ± 10 D; Figure 1A). The amylose from pea embryos appeared to be intermediate in size (peak at 470,210 ± 10 D; Figure 1B) between that in Ia.12 potato starch (peak at 367,320 ± 10 D) and that in Ib.12 potato starch (peak at 655,390 ± 10 D).

These findings suggested that GBSSIa was not the only enzyme that contributed to amylose synthesis in pea embryo. To investigate this further, amylose was concentrated from the *lam* mutant of pea (SIM 503; Denyer et al., 1995), which lacks GBSSIa protein and activity. Gel permeation chromatography revealed that the molecular mass of this amylose (peak at 614,240 ± 0 D) was similar to that of pea leaf, potato Ib.12, and wild-type potato starch (Figures 7C and 7D) and implied that GBSSIb contributes to amylose

synthesis in pea embryos, as does GBSSIa. This conclusion is supported by the presence of a small amount of a 58-kD protein on starch from *lam* pea embryos (Denyer et al., 1997).

By contrast, GBSSIb synthesized most of the amylose in pea leaves. Although GBSSIa was expressed at a low level in pea leaves (Figure 3), the low specific activity of this protein suggests that it makes a very minor contribution to the synthesis of amylose in pea leaves compared with GBSSIb. To confirm that the size of the amylose synthesized was principally a function of the activity of the specific isoform of GBSSI in a particular tissue, amylose from *amf* lines expressing GBSSIa was extracted. The amount of amylose in this leaf starch was extremely low, but it clearly had a low peak molecular mass (370 kD), typical of the product of GBSSIa, as seen in Ia.12 potato tubers (Figure 7E).

The sizes of amyloses from wild-type and GBSSIb potato leaves are shown, for comparison, in the supplementary material (see supplementary data online). No amylose was detected in *amf* leaf starch. Therefore, GBSSIa makes low molecular mass amylose in both tubers and leaves, establishing GBSSI isoform specificity as a major determinant of amylose functionality in higher plants.

Our kinetic data indicated that GBSSIb was very similar to potato GBSSI in its activity and specificity (Tables 1 and 2). Interestingly, the potato amylose from the diploid control line had a relatively high molecular mass, comparable to that synthesized by GBSSIb in the *amf* potato line (Figures 7C and 7D).

## Effects of GBSSI Isoforms on Short Chains of Amylopectin

It has been suggested that GBSSI also contributes to amylopectin biosynthesis, either through the synthesis of intermediates in amylose production (van de Wal et al., 1998) or through a direct contribution to the long chains in amylopectin formation, as suggested for *Chlamydomonas* (Colleoni et al., 1999) and higher plants (McPherson and Jane, 1999).

Fluorophore-assisted gel electrophoresis was used to

Table 4. Starch Synthase Activity on Washed Granules from Wild Type, *amf* plus GBSSIa, and *amf* plus GBSSIb Potato Lines

	Wild Type	Ia.12 (Pea Embryo GBSSIa)	Ib.12 (Pea Leaf GBSSIb)
GBSS activity (nmol·min⁻¹·mg⁻¹ starch)	0.463 ± 0.061	0.074 ± 0.001	0.034 ± 0.006
GBSS activity (% of wild type)	100	16	7
GBSS protein (μg/mg starch)	0.25	0.25	0.001
GBSS protein (% of wild type)	100	100	0.4
Specific activity (μmol·min⁻¹·mg⁻¹ GBSS)	1.85	0.3	3.4
Specific activity (% of potato GBSSI activity in wild type)	100	16	184

Assays were performed on freshly purified, acetone-washed starch from individual tubers of plants late in tuber development. Values are means ± SE from measurements on batches of starch from five separate tubers. For each line, tubers were from at least two separate plants.
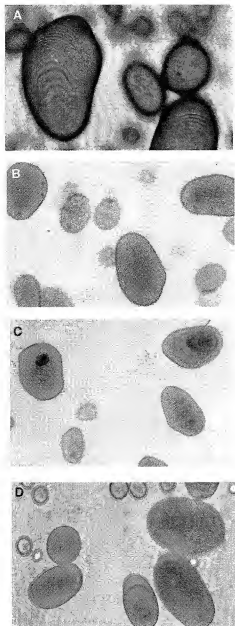
Figure 6. Iodine Staining of Starch Granules.

Starch granules were viewed in a hydrated state in a 10-fold dilution of Lugol's Iodine.
(A) Wild type.
(B) *amf*.
(C) Transgenic line Ia.12.
(D) Transgenic line Ib.12.

examine the chain length distribution of the amylopectin shorter chains (6 to 35 Glc units) after debranching with isoamylase. No significant differences were observed between any of the transgenic lines, the *amf* mutant, the wild type, or the fully complemented mutant BW101 (data not shown). We conclude that GBSSIa and GBSSIb make no contribution to the synthesis of short glucan chains in amylopectin. However, in our hands, fluorophore-assisted gel electrophoresis did not resolve chain lengths with a degree of polymerization >35 with accuracy, so it is possible that these proteins could contribute to the synthesis of long chains in amylopectin.

Physicochemical Analysis of Potato Starches

Starch is used for many applications in food processing and for nonfood industrial uses. These applications of starch depend on its physicochemical properties, which depend on many factors, including the ratio of amylose to amylopectin. The cooking properties of many flours also are determined by the physicochemical characteristics of their starches (Shibanuma et al., 1996; Zeng et al., 1997; Noda et al., 2001), which are correlated closely with the staling properties of bread flours and the cooking quality of noodles (Collado and Corke, 1997; Boggini et al., 2001; Noda et al., 2001). We examined the physicochemical properties of the starches from the potato lines expressing GBSSIa and GBSSIb to determine whether the molecular mass of amylose has a significant impact on the physical properties of starch.

We measured two features, the T_m of the starch granules and the solution properties of the starches, because these parameters are particularly dependent on amylose content. The starches of the wild type, *amf*, *amf* expressing GBSSIa (lines Ia.5 and Ia.12), and *amf* expressing GBSSIb (line Ib.12) were compared. Line Ia.5 was included in this comparison because its amylose content was exactly the same as that of line Ib.12 (0.8%), whereas line Ia.12 had slightly higher amylose composition (1.0%).

Differences in the T_m of the starches were measured by differential scanning calorimetry (DSC). The onset temperature (T_{onset}) for melting of wild-type potato starch was 63.9°C and that of *amf* was 70.3°C, confirming that amylose reduces T_m significantly. The T_{onset} of Ia.5 starch was 70.7°C, that of Ia.5 was 70.5°C, and that of Ib.12 was 67.5°C. These data show that the small amount of amylose present in the Ib.12 starch significantly decreased the T_{onset} for starch melting. However, the equivalent amount of amylose in Ia.5 or slightly more in Ia.12 starch did not shift the T_{onset} significantly from that observed for *amf* starch. These data indicate that the molecular mass of amylose does influence the T_{onset} for melting of starch granules.

The solution properties of the starches were examined using a rheometer, which involves measuring viscosity changes while the starch suspension is heated and then cooled with constant stirring. This type of analysis provides
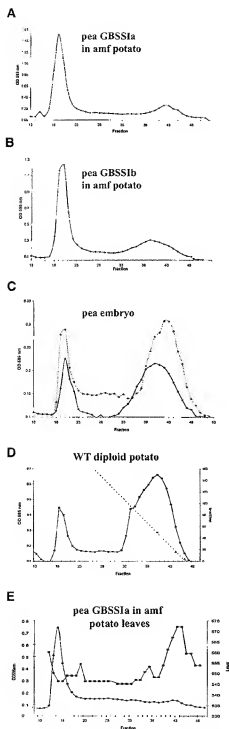
A pea GBSSIa in amf potato

B pea GBSSIb in amf potato

C pea embryo

D WT diploid potato

E pea GBSSIa in amf potato leaves

insight into how the starch may behave as a paste or a sauce. Upon heating, the granules swell and the viscosity of the suspension increases. A point of maximum swelling is reached, known as the peak paste viscosity (Flipse et al., 1996; Visser et al., 1997). After heating and cooling, the starch suspensions are kept at 20°C for 15 min.

During this period, the viscosity increases again, a process known as retrogradation, which is a negative attribute of starches for food uses and for nonfood uses as coatings and paints, because it involves the formation of "skins." Although retrogradation can be strongly influenced by the amylopectin component of starch, it also is correlated positively to increasing amylose content (Visser et al., 1997).

The details of the rheological analysis of wild-type, amf, Ia.5, and Ia.12 starches are shown in Figure 8A. All of the starches started to increase in viscosity at a similar temperature, and the differences in T_{onset} observed in the DSC analysis were not observed using this method. The peak pasting viscosities differed considerably between wild-type and amf starch, but the inclusion of low levels of amylose in lines Ia.5 and Ia.12 reduced the peak pasting viscosity compared with that in amf starch, indicating that this feature is not related linearly to amylose content.

Upon cooling, the setback (the final viscosity of the starch gel) was much higher for wild-type starch than for amf starch, demonstrating the importance of amylose content to retrogradation. The increasing amounts of amylose in Ia.5 and Ia.12 starches increased the setback.

Amylose was partially purified by butanol precipitation, solubilized, and subjected to chromatography on Sepharose CL-2B columns. Fractions eluted from the columns were mixed with an iodine solution, and absorbance was measured at 595 nm (lines).
(A) Amylose from transgenic line GBSSIa.
(B) Amylose from transgenic line GBSSIb.
(C) Amylose from lam pea embryos (the 503 allele, which produces no GBSSIa RNA or protein, was used). For comparison, amylose from wild-type pea embryos is shown as a dotted line.
(D) Amylose from a wild-type (WT) diploid line of potato. The molecular mass of wild-type potato amylose is very similar to that synthesized by GBSSIb in amf potato (B). The regression line for the dextran size markers used to calibrate the column for all samples is shown as a dotted line.
(E) Amylose from leaves of an amf line expressing GBSSIa.
Closed circles indicate the elution profile of glucan determined by iodine staining. Closed squares indicate the wavelength of maximum absorbance of the starch-iodine complex (λ_{max}), and the peak λ_{max} values indicate clearly the size of amylose in this leaf starch. For a comparative analysis of the amylose in total starch preparations from GBSSIa, GBSSIb, wild type, and amf potato leaves, see the supplementary data online. In each case, results from typical experiments are shown.
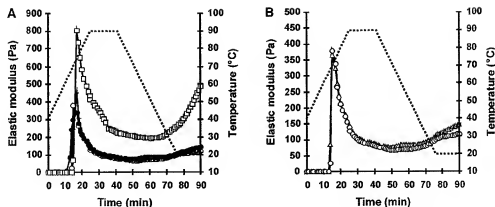
Figure 7. Gel Permeation Chromatography of Partially Purified Amylose from Transgenic amf Tubers, Pea Embryos, Wild-Type Potato, and Potato Leaves.

Figure 8. Changes in the Storage Moduli of 2.5% (w/v) Potato Starch Suspensions during Heating and Cooling (rapid viscometric analysis).

(A) Profiles of starches with increasing amylose contents. Solid line, *amf* starch; open circles, starch from line Ia.5 (0.8% amylose); closed circles, starch from line Ia.12 (1.0% amylose); open squares, wild-type starch; dotted line, temperature.
(B) Profiles from Ia.5 starch with 0.8% amylose of lower molecular mass (open circles) and from Ib.12 starch with 0.8% amylose of higher molecular mass (open triangles); dotted line, temperature.

We compared the starches from lines Ia.5 and Ib.12, which had identical amylose contents (0.8%), to determine the influence of amylose molecular mass on the rheological profiles. These data are shown in Figure 8B. The T_{onset} and the peak pasting viscosities of the two starches were identical, but the setback of Ia.5 starch was lower than that of Ib.12 starch, indicating that the longer amylose produced by GBSSIb is more susceptible to retrogradation than the shorter amylose produced by GBSSIa.

DISCUSSION

Isoforms of GBSSI in Pea

Our analysis of genes encoding GBSSI in pea has demonstrated that at least two genes encode isoforms can catalyze the incorporation of Glc from ADP-Glc into glucan. Both isoforms are tightly bound to starch granules and therefore are granule-bound starch synthases. Their deduced primary amino acid sequences support this interpretation. Despite their structural similarity, these isoforms of GBSSI are not identical. First, they are expressed differentially in plant organs, GBSSIa being expressed predominantly in pea embryos, where storage starch is synthesized, and GBSSIb being expressed predominantly in leaves, where transitory starch is synthesized, although it is expressed in embryos as well (Denyer et al., 1997). This is similar to the situation reported for wheat, in which GBSSI is expressed in endosperm and a second isoform, GBSSII, is expressed in leaves, pericarp, and aleurone (Vrinten and Nakamura, 2000).

The two isoforms of GBSSI from pea also have distinct catalytic properties. GBSSIa activity cannot be measured in vitro using standard assay procedures for starch synthases, whereas GBSSIb activity is detected readily in vitro. Comparison of the kinetic properties of GBSSIb with those of other GBSSI proteins suggests that they are very similar to those of potato GBSSI in terms of its specific catalytic properties and the size of the amylose it synthesizes in vivo. Interestingly, both GBSSIa and GBSSIb are activated upon incorporation into starch granules. The degree of activation is impossible to estimate for GBSSIa because no activity can be detected in vitro (implying a very significant activation upon association with starch granules). For GBSSIb, we estimate activation upon association with the starch granule to be at least 10-fold.

Differences in the Products of GBSSI Isoforms

The two isoforms of GBSSI from pea synthesize distinct forms of amylose. GBSSIa forms a shorter type of amylose than GBSSIb. This is shown quite clearly by comparison of the amylose made in potato *amf* lines transformed with either GBSSIa or GBSSIb. The features of the enzymes that determine product specificity are not clear. It is possible that differences in the affinity of the isoforms for glucans favor the production of shorter amylose by GBSSIa and longer amylose by GBSSIb. Alternatively, the location of

each GBSSI isoform within the starch granule might influence the product it synthesizes. Differences in the location of the amylose synthesized by each isoform across starch granules were apparent from analysis of *amf* transgenic lines.

Differences in the location of the enzymes (or active enzymes) within the granule might influence product specificity through differences in the supply of ADP-Glc substrate (concentration and rate of diffusion) to different places and in the space available for the synthesis of new glucan. The importance of granule association to GBSSIa activity is particularly clear, because no activity of this isoform was detected in vitro. In addition, microheterogeneity within the granule might influence both where this enzyme is active and the specific nature of its product.

Our analysis showed that pea leaves contain a longer form of amylose in their starch granules than do pea embryos. There have been reports of many factors that influence the molecular mass of amylose, including the developmental stage of the tissue (Yun and Matheson, 1992), whether the starch is transitory or storage starch, the relative activity of GBSSI (Fulton et al., 2002), and the particular location of activity within the starch granule (Jane and Shen, 1992). Our data show that the specific isoform of GBSSI also is a major determinant of the molecular mass of amylose.

This specificity in the product of different GBSSI isoforms represents an additional explanation for the differences in the molecular mass of amylose that has not been considered previously. Although GBSSIa is expressed weakly in leaves, its relatively low expression, coupled with its relatively low specific activity (compared with that of GBSSIb), indicates that GBSSIb is the major GBSSI isoform activity in leaves. Embryo starch consists of amylose that is intermediate in size between the product of GBSSIa and that of GBSSIb. This finding indicates that both GBSSIa and GBSSIb operate to synthesize amylose in pea embryos.

Both proteins can be detected in embryos bound tightly to starch (Denyer et al., 1997), and the analysis of starch from *lam* embryos confirms GBSSIb to be present and active, because *lam* (line 503) embryos lack GBSSIa activity yet still make some amylose (estimated at between 4 and 10% total glucan compared with 30% for wild-type embryos) (Wang et al., 1998). This amylose is of higher average molecular mass than that in wild-type embryos and is similar to the product of GBSSIb in potato.

GBSSIa and GBSSIb may be expressed in the same cells of the embryo, and the amylose produced would be the result of their combined specificities. Alternatively, GBSSIa and GBSSIb may be expressed in different cells of the embryo, but upon extraction, the starch granules they synthesize would become mixed. The fact that GBSSIa and GBSSIb synthesize discrete forms of amylose in pea embryos suggests that isoform specificity is a major determinant of amylose molecular mass, at least in the starch of pea embryos.

Our data provide evidence for specificity in starch composition being provided by the differential use of GBSSI isoforms in different tissues of the plant. A recent phylogenetic analysis of the Rosaceae has shown two loci encoding GBSSI in all subfamilies (Evans et al., 2000), suggesting that more than one isoform of GBSSI may be active in some other dicot species, although only one GBSSI gene has been found in the genome sequence of Arabidopsis and only one active form has been identified in potato. In wheat, two structurally distinct isoforms are expressed in different tissues (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000), and there is evidence for at least two genes that encode GBSSI in rice, barley, and maize (Vrinten and Nakamura, 2000).

Based on structural analysis, the GBSSI proteins of cereal storage organs appear quite distinct from those active in dicot storage organs (Figure 2B). These structural differences may imply differences in catalytic activity/specificity, which also could affect product specificity. It has been reported that cereal endosperm starch contains amylose of higher molecular mass than that in pea embryos but lower than that in potato tubers (Gerard et al., 2001). If the cereal endosperm GBSSIs constitute a catalytically distinct subclass of GBSSI proteins, it might be possible to introduce new specificity to amylose production into dicot species such as potato using a cereal GBSSI gene, as we have done with GBSSIa from pea, or, more significantly, to introduce new specificity to amylose production in cereal endosperm using the potato GBSSI or the pea GBSSIb gene.

Interestingly, a GBSSI gene from cassava failed to complement the *amf* mutant in full production of amylose when transformed into this line of potato. These data suggest that cassava GBSSI and potato GBSSI also may be distinct in their activities in plants (Salehuzzaman et al., 1999).

Our experimental analysis of GBSSI isoforms from pea in potato was complicated by the very low levels of expression of GBSSIb we were able to obtain in the *amf* line. This meant that none of the transgenic lines showed full complementation of the *amf* phenotype, despite GBSSIb being a protein with very similar activity and specificity to the potato GBSSI encoded by the *AMF* locus and expressed in potato tubers. We do not know the reason why such low levels of GBSSIb protein were obtained in our transgenic experiments, but we suspect problems as a result of inefficient translation caused by the short leader sequence in the gene construct or differences in codon use between pea and potato, which might have limited GBSSIb protein production, because mRNA levels for the two genes in the different potato lines were equivalent.

However, one advantage of comparing lines with low GBSSIb activity with those expressing GBSSIa is that lines with similar production of amylose were compared despite the very different specific activities of the two isoforms in vivo. We conclude that differences between the amyloses synthesized by the two isoforms resulted from different product

specificities and were not caused by differences in the overall level of GBSSI activity.

Given the facts that GBSSIb is a significantly more active enzyme than GBSSIa, both in vitro and in vivo (i.e., it has a much higher specific activity), and that recent data suggest that the molecular mass of amylose is correlated positively with the level of GBSSI activity in potato (Fulton et al., 2002), comparison of starch products from lines with similar GBSSI activities has been central to establishing the product specificity of the different isoforms. Comparison of starches from lines with equivalent amounts of amylose also has been important in establishing whether or not the distinct forms of amylose synthesized by GBSSIa and GBSSIb confer distinct physical properties on the starches.

Physical Properties of Starches Synthesized by Different GBSSI Isoforms

The amylose composition of the starches affected their melting behavior, as shown by DSC, and their pasting behavior, as shown by rheometer measurements. The T_{onset} for wild-type starch was significantly lower than that of amf starch, which lacks amylose. The starch containing a small amount of amylose synthesized by GBSSIb had an intermediate T_{onset}, whereas the starch containing amylose synthesized by GBSSIa had the same T_{onset} as amf starch. These data suggest that the molecular mass of the amylose does not influence this parameter, because Ia.5 and Ib.12 starches had equivalent amylose contents. However, these differences in T_{onset} were not evident by rheological analysis.

Retrogradation also is a very important feature of starches; it involves the tendency of starches to form skins after heating. Retrogradation can be measured from the viscosity changes that occur upon cooling of starches. Amylose composition is associated strongly with retrogradation, so the wild-type starch increased in viscosity quickly after cooling, whereas the amf starch showed a very slow, low-level increase in viscosity. Significantly, the starch synthesized by GBSSIb (Ib.12) showed a more rapid increase to a higher viscosity than the starch synthesized by GBSSIa (Ia.5) after cooling. These results suggest that the shorter amylose synthesized by GBSSIa may provide beneficial properties to starch pastes in terms of retrogradation.

METHODS

Preparation of Starch

Starch was prepared essentially according to the methods of Edwards et al. (1995) and Tomlinson et al. (1997). Starch was prepared from

the amylose-free (amf) mutant of potato (Solanum tuberosum) (Hovenkamp-Hermelink et al., 1987) and from leaves and tubers of transgenic lines derived from it. Tubers were of comparable sizes when harvested. Leaves were harvested at the end of the day. Starch also was analyzed from a wild-type diploid line (which was formed by doubling up from the haploid progenitor of the amf line [Visser et al., 1989]; the amf line also is diploid after chromosome doubling of the haploid) and the amf mutant transformed with the genomic clone of potato GBSSI (BW101; van der Leij et al., 1991). Starch was prepared from pea (Pisum sativum) embryos and leaves of an inbred round-seeded line (BC1/9RR) and from the low-amylose (lam) line 503, which makes no GBSSIa protein (Denyer et al., 1997).

Starch Analysis

Partial Purification of Amylose Using Butanol

The method of amylose purification was based on that of Schoch (1942). Starch (1.33 g) was heated in 100 mL of distilled water at 100°C until gelatinized. Phosphate buffer (20% [w/v] phosphate solution: 16.4% anhydrous KH_2PO_4 and 3.6% anhydrous K_2HPO_4) was used to adjust the pH to 5.9 to 6.3, and the starch was autoclaved for 1 h at 121°C. The starch then was refluxed for 1 h at 100°C, after which 25 mL of butan-1-ol was added and refluxing was continued for 1 h. The starch was sealed in a 250-mL Duran bottle, placed in a Dewar flask containing boiling water, and left to cool for 36 to 48 h at room temperature. After centrifugation for 30 min at 1800g, crystalline amylose and butanol complexes were collected and dried in an air flow.

Gel Filtration Chromatography

Amylose was dissolved in 1 M NaOH at 13 mg/mL, diluted to 0.35 M NaOH with distilled water, and boiled for 5 min. After cooling, the solubilized amylose was diluted further to 0.2 M NaOH, and 2.4 mL was loaded onto the first of two Sepharose CL-2B columns (15 mm i.d. × 1 m) connected in series, equilibrated, and eluted with 10 mM NaOH. Fractions of 6 mL were collected at a rate of one fraction per 37.5 min. Samples (150 µL) of each fraction were mixed with 50 µL of Lugol's iodine (Sigma) containing 10 mM acetic acid. A_{595} was determined using a microtiter plate reader. The wavelength of maximal absorbance was measured. The columns were calibrated using dextrans of known molecular mass (Sigma).

Chain Lengths of Amylopectin

Short chains of amylopectin (between 6 and 35 Glc units) were analyzed by fluorophore-assisted gel electrophoresis exactly as described by Edwards et al. (1999b). Starch was debranched with isoamylase, labeled with 8-amino-1,3,6-pyrenetrisulfonic acid, and electrophoresed on an Applied Biosystems 373A DNA sequencer (Foster City, CA). Data were collected and analyzed using Genescan 672 software (Applied Biosystems, Foster City, CA). The system was standardized using maltohexaose and maltoheptaose as standards.

Amylose Content

The amylose content of potato tubers was determined as described by Visser et al. (1991).

Iodine Staining

Samples of purified starch granules were suspended in a 10-fold dilution of Lugol's iodine and viewed with a light microscope.

Isolation and Sequencing of cDNA Clones

The isolation and sequence of pea embryo GBSSIa has been described (Dry et al., 1992). To isolate pea leaf GBSSIb cDNA, mRNA was extracted from the leaves of a round-seeded line of pea (BC1/9 RR), and cDNA was synthesized as described by Dry et al. (1992). A λgt10 library was constructed according to the manufacturer's instructions (Amersham International, Amersham, UK). A total of 2 × 10⁴ plaques were probed with a 2.2-kb BamHI fragment containing the full-length cDNA of potato GBSSI (Dry et al., 1992) at 55°C and washed with 2 × SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% (w/v) SDS.

Three positive clones were obtained and subcloned into the EcoRI site of pBluescript SK+, and the sequences were determined. All three clones were full length (2100 bp). The 5' end of the GBSSIb cDNA encodes a peptide sequence that conforms to the consensus sequence for chloroplast transit peptides (Gavel and von Heijne, 1990). The cleavage site for GBSSIb is GIIVC*G, compared with the consensus I/VXa/C*A (where * indicates the point of cleavage). V occupies position −2 (X) in a high proportion of chloroplast transit peptides. There also are characteristic basic residues (K and R) in the −6 to −10 region.

Isolation of RNA, Poly(A)⁺ RNA, Reverse Transcriptase–Mediated PCR, and DNA Gel Blot Analysis

Total RNA was isolated from pea leaves and pea embryos according to the method of Martin et al. (1985), and poly(A)⁺ mRNA was purified using an oligo(dT)-cellulose spin column (Pharmacia). First-strand cDNA was made to 1 μg of poly(A)⁺ RNA primed with a dT(17) adaptor (Frohman et al., 1988) using Superscript reverse transcriptase (Gibco BRL) in accordance with the manufacturer's instructions. The PCR amplification used oligonucleotides of between 26 and 33 bases in length to amplify fragments of 0.9 kb (GBSSIa) and 1.7 kb (GBSSIb). The oligonucleotide pairs were 5'-AATGGCAACAAT-AACGGGATCTTCAATGCCGAC-3' and 5'-TCGGAATTCATCAGG-TAAATTGAGAAGAG-3' for GBSSIa and 5'-GTGACTGCTTCAAGT-AACATAGTATCCAGAAC-3' and 5'-GCAATTCTTGATCATTTCTGC-AAAAG-3' for GBSSIb.

For quantitative reverse transcriptase–mediated PCR, 1 μL of cDNA was amplified as described by Frohman et al. (1988), and samples were taken after 15, 20, 25, 30, 35, and 40 cycles to determine the linear range for amplification. PCR was performed with 30 cycles, and the DNA was separated on 1% agarose gels, blotted onto nitrocellulose, and probed with isoform-specific cDNA fragments (200 bp) prepared as described by Edwards et al. (1995). The oligonucleotide pairs used to generate the probes were 5'-AATGGCAAC-

AATAACGGGATCTTCAATGCCGAC-3' and 5'-CAATGACTTTTC-ACTGGTATCTGAAGAACCG-3' for GBSSIa and 5'-GTGACTGCT-TCAAGTAACATAGTATCCAGAAC-3' and 5'-CTAACATTCTTTCTC-TGGACACCCTTCTTCC-3' for GBSSIb. Control reactions contained plasmid DNA containing full-length GBSSIa and GBSSIb cDNA (pGBSSIa and pGBSSIb), respectively.

Construction of Expression Plasmids for Mature GBSSIa and GBSSIb

PCR mutagenesis was used to introduce ATG initiation codons at the 5' end of the mature cDNAs and to optimize codon use for the first nine amino acids. The sequences of the oligonucleotides were as follows: GBSSIa, 5'-GAATTCCATGGGTCTGGTTTCGTTGGTGCTGA-AGTTGGACCTTGG-3'; GBSSIb, 5'-GTTTCCATGGTGAATTTGATC-TTTGTTGGAACTGAGG-3'.

The expression constructs were prepared as in-frame S-tag fusions between the NcoI-BamHI sites (GBSSIa) or NcoI-EcoRI sites (GBSSIb) of pStag (Edwards et al., 1999a) to give plasmids pStag19 and pStag46, respectively. Recombinant proteins were synthesized with a 15–amino acid N-terminal tag, the S-peptide, which was used for quantification and detection on protein gel blots (Kim and Raines, 1993). The presence of the S-tag has been shown not to influence potato GBSSI activity in solution (Edwards et al., 1999a).

Expression of GBSSIa and GBSSIb in Escherichia coli

Recombinant proteins were expressed in E. coli RH98 (a glycogen synthase mutant) exactly as described by Edwards et al. (1999a). Host cells also contained plasmids pLysE and pSBET for improved expression (Studier, 1991; Schenk et al., 1995). Protein expression was induced by the addition of isopropylthio-β-galactoside to a final concentration of 1 mM. Cells were harvested, resuspended in extraction buffer (100 mM Mops, pH 7.2, 5 mM MgCl₂, 5% [v/v] glycerol, and 2 mM DTT), and lysed using a French pressure cell. Soluble and insoluble fractions were separated by centrifugation at 4°C for 10 min at 10,000g, and the soluble fractions were used for further analysis.

SDS-PAGE and Immunoblot Analysis

Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized using either anti-GBSSI antiserum (Smith, 1990) followed by alkaline phosphatase–conjugated goat anti-rabbit serum (Sigma). For quantification of GBSSI protein expressed in transgenic potato, starch-granule bound proteins were separated by SDS-PAGE, visualized with Coomassie brilliant blue R 250, and quantified by reference to BSA standards run on the same gel.

Starch Synthase Assays

All reactions had a final volume of 100 μL, and the buffer consisted of 100 mM Bicine, pH 8.5, 25 mM potassium acetate, 5 mM EDTA, and

10 mM DTT. Unless stated otherwise, 0.23 kBq of ADP-U-¹⁴C-Glc was used per 100-µL reaction. Assays were incubated at 25°C for 20 min, terminated by heating at 90°C for 2 min, and processed by either the methanol/KCl method or the resin method (Denyer et al., 1999). All assays and controls were duplicated.

Bacterial Extracts

Assays were performed as described by Edwards et al. (1999a). Kinetic constants were determined using potato amylopectin at concentrations up to 80 mg/mL and ADP-Glc concentrations up to 2 mM. Specific activity measurements were made with the following: 5 mg/mL amylopectin; 50 mg/mL amylopectin; 5 mg/mL amylopectin and 0.5 M citrate; 50 mg/mL amylopectin and 0.5 M citrate; 100 mM maltotriose; 100 mM maltotriose and 5 mg/mL amylopectin.

GBSSIa (pStag19) also was assayed in the presence of the following: 200 mg/mL amylopectin; 10 mg/mL glycogen; 25% (w/v) glycerol; 5 and 25 mg/mL amylose-free potato starch; perchloric acid-treated amylose-free starch granules; 1 mM UDPG; debranched (isoamylase-treated) amylopectin; 10 mg/mL amylose (solubilized by sonication and boiling); 10 mg/mL amylose and 100 mM maltotriose; 100 mM maltooligosaccharides from G3 to G7; 60 mM ADP-Glc; no DTT; E. coli chaperonins, GROESL, GROE, GROS, and GROL on one plasmid (Gatenby et al., 1990); and after preincubation overnight at 25°C with and without amylopectin.

Processivity assays were performed in the presence of 1 mM ADP-Glc, 100 mM maltotriose, 2.5 mg/mL amylopectin, and 1.84 kBq ADP-U-¹⁴C-Glc. The reactions were allowed to proceed for 1 h at 25°C, and the products were processed using the resin method and analyzed by high-performance anion-exchange chromatography as described previously (Edwards et al., 1999a).

Potato Starch Granules

Starch was resuspended in extraction buffer (100 mM Mops, pH 7.2, 1 mM EDTA, 10% [v/v] ethanediol, and 1 mM DTT) at a concentration of 20 to 40 mg/mL. Assays contained 50 µL (1 to 2 mg) of starch, 1 mM ADP-Glc, 5 mg/mL amylopectin, and 100 mM maltotriose and were processed by the methanol/KCl method (Denyer et al., 1999).

Processivity assays were performed as described above except that they contained 40 to 80 mg/mL of starch granules and 1.84 kBq of ADP-U-¹⁴C-Glc, were incubated for 1 h at 25°C, and were not boiled to terminate. Reaction products were processed using the resin method and analyzed by high-performance anion-exchange chromatography as described previously (Edwards et al., 1999a).

Construction of Sense Binary Vectors and Transformation of amf Potato

A 2.0-kb fragment encoding full-length embryo GBSSIa and a 1.8-kb fragment encoding full-length leaf GBSSIb were subcloned in the sense orientation between the double 35S promoter of Cauliflower mosaic virus (CaMV) and the CaMV terminator in pJIT60 (Guerineau and Mullineaux, 1993), producing plasmids pGBSSIa and pGBSSIb, respectively. The KpnI-XhoI fragment from pGBSSIa and the SstI-XhoI fragment from pGBSSIb, both encompassing the double 35S

promoter, cDNA, and CaMV terminator, were ligated into the plant transformation vector pBIN19 (Bevan, 1984), resulting in plasmids pBinGBSSIa (embryo GBSSIa) and pBinGBSSIb (leaf GBSSIb). In vitro-grown stem segments were used for the transformation of the potato amf mutant. Agrobacterium tumefaciens-inoculated stem explants were transferred to callus-induction and shoot-regeneration medium as described by Visser (1991).

Measurement of Physicochemical Properties

Determination of the T_m of Starch Granules

The temperature at which starch granules started to gelatinize was determined by differential scanning calorimetry using a Perkin-Elmer Pyris 1 calorimeter equipped with a ThermoNeslab RTE-140 glycocooler (Portsmouth, NH). The instrument was calibrated with indium (T_m of 156.6°C) and zinc (T_m of 419.47°C). Before differential scanning calorimetry analysis, the moisture content of starch samples was determined by drying them overnight in an oven at 105°C and weighing them before and after drying.

Precisely 10 mg of starch was transferred to a stainless-steel pan, and the moisture content of the pan was adjusted to 20% by adding an appropriate amount of water. The pan was sealed and allowed to equilibrate overnight. The samples were heated from 40 to 100°C at a scanning rate of 10°C/min. An empty sample pan was used as a reference. For each endotherm, the onset temperature of gelatinization and the difference in enthalpy were computed automatically.

Determination of the Solution Properties of Transgenic Starches

Dynamic rheological properties of 2.5% (w/v) starch suspensions at small deformations were determined by applying a small oscillating shear deformation using a Bohlin CVO rheometer (Mettler Toledo, Tiel, The Netherlands). The apparatus was equipped with a stainless-steel small sample cell with the following geometric parameters: cylinder i.d. of 25 mm, cylinder o.d. of 26 mm. The torque bar was adjusted automatically by the machine based on an initial stress of 0.019 Pa, a target strain of 0.100, and an oscillation frequency of 0.1 Hz. The suspensions were preheated to ~40°C under gentle stirring and loaded in the sample cell (preheated at 40°C). After this, the cell was subjected to the following temperature program: heating to 90°C, 15 min at 90°C, cooling to 20°C, and 15 min at 40°C. Heating and cooling were performed at a rate of 2°C/min. Data were collected automatically every 10 s.

Accession Numbers

The accession numbers for the plants described in Figure 2B are as follows: barley (Hordeum vulgare), X07932 (Rohde et al., 1988); wheat (Triticum aestivum), X57233 (Clark et al., 1991); maize (Zea mays), X03935 (Klosgen et al., 1986); sorghum (Sorghum bicolor), Q43134 (Y.C. Hsing, unpublished data); rice (Oryza sativa), X62134 (R.J. Okayaki, unpublished data); bean (Phaseolus vulgaris), AB029546 (N. Isono, K. Nozaki, H. Ito, H. Matsui, and M. Honma, un-

published data); peala (*Pisum sativum*), X88789 (Dry et al., 1992); Antirrhinum (*Antirrhinum majus*), AJ006293 (Mérida et al., 1999); potato (*Solanum tuberosum*), X58453 (van der Leij et al., 1991); sweet potato (*Ipomoea batatas*), U44126 (S.-J. Wang, K.-W. Yeh, and C.-Y. Tsai, unpublished data); cassava (*Manihot esculenta*), X74160 (Salehuzzaman et al., 1993); pealb (*P. sativum*), AJ345045 (this article); Arabidopsis (*Arabidopsis thaliana*), O49727 (M. Bevan, G. Murphy, L. Drost, C. Hall, S. Hudson, P. Ridley, I. Bancroft, H.W. Mewes, K. Mayer, and C. Schueller, unpublished data) and wheatII (*T. aestivum*), AF109395 (Vrinten and Nakamura, 2000). The accession number for the full-length cDNA of potato GBSSI (Dry et al., 1992) is X87988.

REFERENCES

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12,** 8711–8721.

Boggini, G., Cattaneo, M., Paganoni, C., and Vaccino, P. (2001). Genetic variation for waxy proteins and starch properties in Italian wheat germplasm. Euphytica **119,** 111–114.

Clark, J.R., Robertson, M., and Ainsworth, C.C. (1991). Nucleotide sequence of a wheat (*Triticum aestivum* L.) cDNA clone encoding the waxy protein. Plant Mol. Biol. **16,** 1099–1101.

Collado, L.S., and Corke, H. (1997). Properties of starch noodles as affected by sweetpotato genotype. Cereal Chem. **74,** 182–187.

Colleoni, C., et al. (1999). Genetic and biochemical evidence for the involvement of α-1,4-glucotransferases in amylopectin synthesis. Plant Physiol. **120,** 993–1003.

Commuri, P.D., and Keeling, P.L. (2001). Chain-length specificities of maize starch synthase I enzyme: Studies of glucan affinity and catalytic properties. Plant J. **25,** 475–486.

Craig, J., Lloyd, J.R., Tomlinson, K., Barber, L., Edwards, A., Wang, T.L., Martin, C., Hedley, C.L., and Smith, A.M. (1998). Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. Plant Cell **6,** 413–426.

Denyer, K., Barber, L.M., Burton, R., Hedley, C.L., Hylton, C.M., Johnson, S., Jones, D.A., Marshall, J., Smith, A.M., Tatge, H., Tomlinson, K., and Wang, T.L. (1995). The isolation and characterization of novel low-amylose mutants of *Pisum sativum* L. Plant Cell Environ. **18,** 1019–1026.

Denyer, K., Barber, L.M., Edwards, E.A., Smith, A.M., and Wang, T.L. (1997). Two isoforms of the GBSSI class of granule-bound starch synthase are differentially expressed in the pea plant (*Pisum sativum* L.). Plant Cell Environ. **20,** 1566–1572.

Denyer, K., Waite, D., Edwards, A., Martin, C., and Smith, A.M. (1999). Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. Biochem. J. **342,** 647–653.

Devereux, J., Haberli, P., and Smithies, O. (1984). A comprehensive set of sequence analyses for the VAX. Nucleic Acids Res. **12,** 387–395.

Dry, I., Smith, A., Edwards, A., Bhattacharrya, M., Dunn, P., and Martin, C. (1992). Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. Plant J. **2,** 193–202.

Edwards, A., Borthakur, A., Bornemann, S., Venail, J., Denyer, K., Waite, D., Fulton, D., Smith, A., and Martin, C. (1999a). Specificity of starch synthase isoforms from potato. Eur. J. Biochem. **266,** 724–736.

Edwards, A., Fulton, D., Hylton, C., Jobling, S., Gidley, M., Rossner, U., Martin, C., and Smith, A.M. (1999b). A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. Plant J. **17,** 251–262.

Edwards, A., Marshall, J., Sidebottom, C., Visser, R.G.F., Smith, A.M., and Martin, C. (1995). Biochemical and molecular characterization of a novel starch synthase from potato tubers. Plant J. **8,** 283–294.

Evans, R.C., Alice, L.A., Campbell, C.S., Kellogg, E.A., and Dickinson, T.A. (2000). The granule-bound starch synthase (GBSSI) gene in the Rosaceae: Multiple loci and phylogenetic utility. Mol. Phylogenet. Evol. **17,** 388–400.

Flipse, E., Suurs, L., Keetels, C.J.A.M., Kossmann, J., Jacobsen, E., and Visser, R.G.F. (1996). Introduction of sense and antisense cDNA for branching enzyme in the amylose-free potato mutant leads to physico-chemical changes in the starch. Planta **198,** 340–347.

French, D. (1984). Organization of starch granules. In Starch: Chemistry and Technology, 2nd ed, R.L. Whistler, J.N. Bermiller, and E.F. Paschall, eds (New York: Academic Press), pp. 183–247.

Frohman, M.A., Dush, M.K., and Martin, G.R. (1988). Rapid amplification of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85,** 8998–9002.

Fujita, N., and Taira, T. (1998). A 56-kDa protein is a novel granule-bound starch synthase existing in the pericarps, aleurone layers and embryos of immature seeds in diploid wheat (*Triticum monococcum* L.). Planta **207,** 125–132.

Fulton, D.C., Edwards, A., Pilling, E., Robinson, H.L., Fahy, B., Seale, R., Kato, L., Donald, A.M., Geigenberger, P., Martin, C., and Smith, A.M. (2002). Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. J. Biol. Chem. **277,** 10834–10841.

Gatenby, A.A., Viitanen, P.V., and Lorimer, G.H. (1990). Chaperonin assisted polypeptide folding and assembly: Implications for the production of functional proteins in bacteria. Trends Biotechnol. **8,** 354–358.

Gavel, Y., and von Heijne, G. (1990). A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett. **261,** 455–458.

Gerard, C., Barron, C., Colonna, P., and Planchot, V. (2001).

Amylose determination in genetically-modified starches. Carbo-hydr. Polym. 44, 19–27.

Guerineau, F., and Mullineaux, P. (1993). Plant transformation and expression vectors. In Plant Molecular Biology Labfax, R.R.D. Croy, ed (Oxford, UK: BIOS Scientific), pp 121–148.

Hizukuri, S., and Takagi, T. (1984). Estimation of the distribution of molecular weight for amylose by the low-angle-light-scatter-ing technique combined with high-performance gel chromatogra-phy. Carbohydr. Res. 134, 1–10.

Hovenkamp-Hermelink, J.H.M., Jacobsen, E., Ponstein, A.S., Visser, R.G.F., Vos-Scheperkeuter, G.H., Bijmolt, E.W., de Vries, J.N., de Witholt, B., and Feenstra, W.J. (1987). Isolation of an amylose-free mutant of the potato (Solanum tuberosum L). Theor. Appl. Genet. 75, 217–221.

Impari-Radosevich, J.M., Nichols, D.J., Li, P., McLean, A.L., Keeling, P.L., and Guan, H.P. (1999). Analysis of purified starch synthases IIa and IIb SS isoforms can be distinguished based on their kinetic properties. Arch. Biochem. Biophys. 362, 131–138.

Ishikawa, N., Ishihara, J., and Itoh, M. (1994). Artificial induction and characterization of amylose-free mutants of barley. Barley Genet. Newsl. 24, 49–53.

Jane, J., and Shen, J.J. (1992). Internal structure of the potato starch granule revealed by chemical gelatinisation. Carbohydr. Res. 247, 279–290.

Kim, J.S., and Raines, R.T. (1993). Ribonuclease S-peptide as a carrier in fusion proteins. Protein Sci. 2, 348–356.

Klosgen, R.B., Gierl, A., Schwarz-Sommer, Z.S., and Saedler, H. (1986). Molecular analysis of the waxy locus of Zea mays. Mol. Gen. Genet. 203, 237–244.

Lloyd, J.R., Landschutze, V., and Kossman, J. (1999). Simulta-neous antisense inhibition of two starch synthase isoforms in potato tubers leads to accumulation of grossly modified amy-lopectin. Biochem. J. 338, 515–521.

Martin, C., Carpenter, R. Sommer, H., Saedler, H. and Coen, E.S. (1985). Molecular analysis of instability in flower pigmentation in Antirrhinum majus following isolation of the pallida locus by trans-poson tagging. EMBO J. 4, 1625–1630.

Mason-Gamer, R.J., Weil, C.F., and Kellogg, E.A. (1998). Granule-bound starch synthase: Structure, function, and phylogenetic util-ity. Mol. Biol. Evol. 15, 1658–1673.

Matheson, N.K., and Wheatley, J.M. (1962). Starch changes in developing and senescing tobacco leaves. Aust. J. Biol. Sci. 15, 445–458.

McPherson, A.E., and Jane, J. (1999). Comparison of waxy potato with other root and tuber starches. Carbohydr. Polym. 40, 57–70.

Mérida, A., Rodriguez-Galan, J.M., Vincent, C., and Romero, J.M. (1999). Expression of the granule-bound starch synthase I (Waxy) gene from snapdragon is developmentally and circadian clock regulated. Plant Physiol. 120, 401–409.

Murata, T., Sugiyama, T., and Akazawa, T. (1965). Enzymatic mechanism of starch synthesis in glutinous rice grains. Biochem. Biophys. Res. Commun. 18, 371–376.

Nakamura, T., Vrinten, P., Hayakawa, K., and Ikeda, J. (1998). Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol. 118, 451–459.

Nakamura, T., Yamamori, M., Hirano, H., Hidaka, S., and Nagamine, T. (1995). Production of waxy (amylose-free) wheats. Mol. Gen. Genet. 248, 253–259.

Noda, T., Tohnooka, T., Taya, S., and Suda, I. (2001). Relationship between physico-chemical properties of starches and white
salted noodle quality in Japanese wheat flours. Cereal Chem. 78, 395–399.

Rohde, W., Becker, D., and Salamini, F. (1988). Structural analysis of the waxy locus from Hordeum vulgare. Nucleic Acids Res. 16, 7185–7186.

Salehuzzaman, S.N., Jacobsen, E., and Visser, R.G. (1993). Isola-tion and characterization of the cDNA encoding granule-bound starch synthase in cassava (Manihot esculenta Crantz) and its antisense expression in potato. Plant Mol. Biol. 23, 947–962.

Salehuzzaman, S.N.I.M., Vincken, J.P., van de Wal, M., Straatman-Engelen, I., Jacobsen, E., and Visser, R.G.F. (1999). Expression of a cassava granule-bound starch synthase in the amylose-free potato only partially restores amylose content. Plant Cell Environ. 22, 1311–1318.

Schenk, P.M., Mattes, R., and Steinbiss, H.H. (1995). Improved high-level expression system for eucaryotic genes in Escherichia coli using T7 RNA polymerase and rare Arg tRNAs. Biotechniques 19, 196–200.

Schoch, T.J. (1942). Fractionation of starch by selective precipita-tion with butanol. J. Am. Chem. Soc. 64, 2957–2961.

Shannon, J.C., and Garwood, D.L. (1984). Genetics and physiol-ogy of starch development. In Starch: Chemistry and Technology, 2nd ed, R.L. Whistler, J.N. Bermiller, and E.F. Paschall, eds (New York: Academic Press), pp. 26–86.

Shibanuma, Y., Takeda, Y., and Hizukuri, S. (1996). Molecular and pasting properties of some wheat starches. Carbohydr. Polym. 29, 253–261.

Smith, A.M. (1990). Evidence that the 'waxy' protein of peas (Pisum sativum) is not the major starch-granule-bound starch synthase. Planta 182, 599–604.

Smith, A.M., Denyer, K., and Martin, C. (1997). The synthesis of the starch granule. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 67–87.

Stimmer, K., and von Haeseler, A. (1996). Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topol-ogies. Mol. Biol. Evol. 13, 964–969.

Stimmer, K., and von Haeseler, A. (1997). Likelihood mapping: A simple method to visualize phylogenetic content of a sequence alignment. Proc. Natl. Acad. Sci. USA 94, 6815–6819.

Studier, F.W. (1991). Use of bacteriophage T7 to improve an induc-ible T7 expression system. J. Mol. Biol. 219, 37–44.

Taira, T., Vermatsu, M., Nakano, Y., and Morikawa, T. (1991). Molecular comparison and identification of the starch synthase bound to starch granules between endosperm and leaf blades in rice plants. Biochem. Genet. 29, 302–311.

Tomlinson, K.L., Lloyd, J.R., and Smith, A.M. (1997). Importance of isoforms of starch-branching enzyme in determining the struc-ture of starch in pea leaves. Plant J. 11, 31–43.

van der Leij, F.R., Visser, R.G.F., Ponstein, A.S., Jacobsen, E., and Feenstra, W.J. (1991). Sequence of the structural gene for granule-bound starch synthase of potato (Solanum tuberosum L.) and evidence for a single point deletion in the amf allele. Mol. Gen. Genet. 228, 240–248.

van de Wal, M., D'Hulst, C., Vincken, J.P., Buléon, A., Visser, R., and Ball, S. (1998). Amylose is synthesised in vitro by extension and cleavage from amylopectin. J. Biol. Chem. 273, 22232–22240.

Visser, R.G.F. (1991). Regeneration and transformation of potato by Agrobacterium tumefaciens. In Plant Tissue Culture Manual, Vol. B5, K. Lindsey, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp 1–9.

Visser, R.G.F., Jacobsen, E., Hesseling-Meinders, A., Schans, M.J., Witholt, B., and Feenstra, W.J. (1989). Transformation of homozygous diploid potato with an *Agrobacterium tumefaciens* binary vector system by adventitious shoot regeneration on leaf and stem segments. Plant Mol. Biol. **12,** 329–337.

Visser, R.G.F., Somhorst, I., Kuipers, G.J., Ruys, N.J., Feenstra, W.J., and Jacobsen, E. (1991). Inhibition of expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol. Gen. Genet. **255,** 289–296.

Visser, R.G.F., Suurs, L.C.J.M., Steeneken, P.A.M., and Jacobsen, E. (1997). Some physicochemical properties of amylose-free potato starch. Starch **49,** 443–448.

Vrinten, P.L., and Nakamura, T. (2000). Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol. **122,** 255–263.

Wang, T., Bogracheva, T.Y., and Hedley, C.L. (1998). Starch: As simple as A, B, C? J. Exp. Bot. **49,** 481–502.

Weatherwax, P. (1922). A rare carbohydrate in waxy maize. Genetics **7,** 568–572.

Yun, S.H., and Matheson, N.K. (1992). Structural changes during development in the amylose and amylopectin fractions (separated by precipitation with concanavalin A) of starches from maize genotypes. Carbohydr. Res. **270,** 85–101.

Zeng, M., Morris, C.F., Batey, I.L., and Wrigley, C.W. (1997). Sources of variation for starch gelatinization, pasting and gelation properties in wheat. Cereal Chem. **74,** 63–71.

Expression of a cassava granule-bound starch synthase gene in the amylose-free potato only partially restores amylose content

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ABSTRACT

Granule-bound starch synthase I (GBSS I) is responsible for the synthesis of amylose in starch granules. A heterologous cassava GBSS I gene was tested for its ability to restore amylose synthesis in amylose-free (*amf*) potato mutants. For this purpose, the cassava GBSS I was equipped with different transit peptides. In addition, a hybrid containing the potato transit peptide, the N-terminal 89 amino acids of the mature potato GBSS I, and the C-terminal part of cassava GBSS I was prepared. The transgenic starches were first analysed by iodine staining. Only with the hybrid could full phenotypic complementation of the *amf* mutation be achieved in 13% of the plants. Most transformants showed partial complementation, but interestingly the size of the blue core was similar in all granules derived from one tuber of a given plant. The amylose content was only partially restored, up to 60% of wild-type values or potato GBSS I-complemented plants; however, the GBSS activity in these granules was similar to that found in wild-type ones. From this, and the observation that the hybrid protein (a partial potato GBSS I look-alike) performs best, it was concluded that potato and cassava GBSS I have different intrinsic properties and that the cassava enzyme is not fully adapted to the potato situation.

Key-words: Solanum tuberosum; amylose; granule-bound starch synthase I; heterologous expression.

INTRODUCTION

Many crops store their carbohydrate as starch. This reserve material consists of a linear glucan called amylose and a branched glucan, amylopectin. In amylose, D-Glc$p$ residues are connected by α-(1 → 4) linkages. Amylopectin is built up from a collection of α-(1 → 4)-D-glucan chains which are connected in a cluster-like fashion by α-(1 → 6) linkages.

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Starch from potato and cassava contains about 20% amylose and 80% amylopectin (Smith *et al.* 1997).

Whereas amylopectin biosynthesis seems to be the result of concerted action of many enzymes, amylose can be produced by the action of predominantly one enzyme, granule-bound starch synthase I (GBSS I). Depending on the conditions under which amylose biosynthesis proceeds, both maltodextrins (Denyer *et al.* 1996) and amylopectin (van de Wal *et al.* 1998) can be used as a primer for GBSS I. Down-regulation of this enzyme activity by either mutation or antisense technology can reduce the amount of amylose completely. For instance, the amylose-free mutant of potato (referred to as *amf* potato) is a recessive monogenic mutation, which is expressed in all starch-containing generative (pollen) and vegetative tissues (collumella cells of root tips, guard cells of stomata, tubers) (Hovenkamp-Hermelink *et al.* 1987). In this mutant, a GBSS I transcript is still formed, but due to a point deletion in the sequence encoding the transit peptide, the GBSS I protein is targeted improperly to the amyloplast (van der Leij *et al.* 1991a). The potato genomic GBSS I gene is able to fully complement the mutant (van der Leij *et al.* 1991b; Flipse *et al.* 1994). In the present paper, the *amf* mutant is used as an experimental system to (i) investigate whether the mutation can be complemented with a foreign GBSS I cDNA from cassava (meGBSS I; *Manihot esculenta*) (Salehuzzaman *et al.* 1993), and (ii) compare the performance of cassava GBSS I with that from potato (stGBSS I; *Solanum tuberosum*).

MATERIALS AND METHODS

Plant materials

The diploid (2 n = 2 x = 24) homozygous amylose-free (*amf*) potato mutant 87·1029-31 (*Solanum tuberosum* L. Jacobsen *et al.* 1989) was propagated *in vitro* from stem segments on Murashige/Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 30 g dm^{-3} sucrose and 8 g dm^{-3} agar (referred to as MS30). Transgenic shoots were similarly grown and multiplied on MS30 medium containing 100 mg dm^{-3} kanamycin.

Construction of chimaeric GBSS I genes

Four different constructs were made which carried either the entire meGBSS I cDNA (Salehuzzaman *et al.* 1993) or hybrids of the meGBSS I cDNA and stGBSS I gDNA (Van der Leij *et al.* 1991a), inserted between the (tuber-specific) potato GBSS I promoter (Visser *et al.* 1991) and the nopaline synthase terminator. In order to assemble the chimeric constructs it was necessary to introduce and remove a number of restriction sites. In stGBSS I gDNA, the following changes were made: introduction of a *Hind*III site (nt –656 to –661), an *Nco*I site (nt –2 to 4) and two *Sph*I sites (nt 230–235 and 665–670), as well as a removal of a *Hind*III site (nt 130–135). In meGBSS I, three *Sph*I sites (nt –2 to 4, 233–238, and 500–505) were introduced. The nt numbers refer to the position in the published sequences. All constructs were sequenced to confirm their correctness. The constructs were first assembled in pUC19, and subsequently subcloned between the *Hind*III and *Eco*RI sites of the plant transformation vector, pBin19 (Bevan 1984). The actual GBSS I proteins produced by these constructs, as well as amino acid modifications due to the cloning strategy are discussed in detail in Results. The binary vectors were transferred to *Agrobacterium tumefaciens* strain LBA 4404 using the direct transformation procedure described by Höfgen & Willmitzer (1988). The integration of the plasmid in *A. tumefaciens* was confirmed by analysing the plasmid DNA isolated from the transformed *A. tumefaciens* strains (Holmes & Quigley 1981).

Transformation and tuber induction

Transformation of potato material was done by co-cultivation of the stem explants in the *A. tumefaciens* suspension for a few minutes, and subsequent transfer to callus induction and shoot regeneration medium consisting of MS20 with 1 mg dm^{-3} zeatin, 200 mg dm^{-3} cefotaxime, 200 mg dm^{-3} vancomycin and 100 mg dm^{-3} kanamycin (Visser 1991). The stem segments were transferred to fresh medium every 3 weeks until shoot outgrowth took place after 3–4 weeks. These shoots were further subcultured at least twice in MS30 with 100 mg dm^{-3} kanamycin. In this selection medium, only the transgenic shoots were able to form roots. Micro-tubers were induced from transgenic shoots by transferring stem pieces with a nodal bud to MS80 medium containing 2 mg dm^{-3} BAP and culturing them in the dark. Starch from micro- and greenhouse-grown tubers was isolated according to Salehuzzaman *et al.* (1993) and immediately stored at –20 °C.

Immunoblotting, copy number determination and RNA analysis

GBSS protein was extracted by boiling 20 mg starch in 200 mm^3 extraction buffer (Salehuzzaman *et al.* 1993). After centrifugation (2 min, 20 000 g), the supernatants were run in a 10% polyacrylamide gel and immunoblotting was

carried out as described by Hovenkamp-Hermelink *et al.* (1987) using antiserum raised against potato GBSS I (Vos-Scheperkeuter *et al.* 1986).

DNA was extracted from the leaves of transgenic plants according to Dellaporta *et al.* (1983) and digested with *Hind*III, which does not cut in any of the constructs. ^{32}P random-prime-labelled meGBSS I cDNA was used as probe and the minimum number of T-DNA inserts was determined by scoring the number of additional bands in Southern blots.

RNA isolation and DNA/RNA hybridization was performed as described before (Salehuzzaman *et al.* 1993, 1994) using potato and cassava GBSS I DNA as a probe. Equal amounts of RNA were loaded on the gel, which was checked by hybridizing RNA blots with a ribosomal DNA probe (not shown).

Analytical methods

Determination of amylose content

The amount of amylose was determined qualitatively by staining the starch granules with a 1:1 mixture of water and Lugol solution (I$_2$-KI, Jacobsen *et al.* 1989), and investigating these granules visually with a microscope. For quantitative determination of the amylose content, 2 mg of starch was treated with perchloric acid, subsequently stained with Lugol solution, and finally the absorbance was measured at 618 nm and 550 nm (Hovenkamp-Hermelink *et al.* 1988).

Determination of GBSS activity

GBSS activity was measured by incubating approximately 2 mg of enzymatically active starch granules with ^{14}C-labelled ADP-Glc (as donor substrate) and determining the incorporation of [^{14}C]ADP-Glc after 30 min and 90 min at 37 °C (Vos-Scheperkeuter *et al.* 1986). The GBSS activity was calculated from the difference in incorporation.

Determination of physical characteristics

Differential scanning calorimetry and dynamic rheological properties of 8% starch suspensions at small deformations were determined essentially as described by Flipse *et al.* (1996a).

Chain-length distribution

Starch samples (2% w/v) were dissolved at 120 °C in dH$_2$O. The pH of the warm solution was adjusted to 3·5, and subsequently the starch was debranched with isoamylase (Hayashibara; 0·66 mg enzyme per cm^3 starch solution) at 40 °C for 2 h, after which the enzyme was inactivated at 100 °C for 30 min. After cooling to room temperature, the samples were diluted twice with 0·1 m NaOH, and subsequently filtered through a 0·2 μm filter. The chain-length distribution was determined as described by Kortstee *et al.* (1996).

RESULTS

Preparation of constructs

Four different constructs were made to investigate whether a foreign GBSS I cDNA from cassava could restore amylose biosynthesis in an *amf* background in potato. The proteins produced with the different constructs are schematically shown in Fig. 1. The cassava GBSS I was equiped with various transit peptides. The c↓CC and p↓CC constructs contained the transit peptides, respectively cassava and potato GBSS I, whereas in p↓c↓CC both transit peptides were fused without destroying (in principle) the two splice sites (c is derived from meGBSS I, p from stGBSS I; normal letters denote transit peptide, capital letters the mature peptide; vertical arrow indicates splice site). In addition to these, a construct p↓PC was also made, in which the first 89 amino acids of the mature meGBSS I (containing the putative ADP-Glc binding site) were exchanged with the corresponding sequence from its potato homologue. As a result of this sequence swapping, a few amino acids were modified. In p↓CC, the first amino acid of the mature meGBSS I was changed (G1M), whereas in p↓c↓CC only the second amino acid of the cassava transit peptide was altered (A2P). In p↓PC, a P89R (in the potato sequence) and a F91L (in the cassava sequence) mutation were introduced, yielding an amino acid sequence of FVDH*RML*LEKV in the hybrid protein (modified amino acids are in italic). Next to the (hybrid) proteins containing (part of) meGBSS I, the full-length stGBSS I (p↓PP) is also included in Fig. 1.

Restoration of amylose synthesis in the *amf* potato by cassava GBSS I cDNA

Tuber starch from transgenic *amf* potato plants containing one of the above constructs was stained with iodine to detect restoration of amylose synthesis. In many cases, the starch granules contained a blue core of varying size, indi-cating partial restoration of amylose synthesis by a foreign GBSS I (cassava) in our model system (the *amf* potato). The size of the blue core was variable between starch granules of one transformant carrying the same construct. This clearly indicated that amylose synthesis was not restored to a similar degree in all transformants. Moreover, starch granules of some transformants still stained completely red (like the *amf* mutant) showing absence of complementation.

Within each transformant, there was only little variability in the phenotype of the starch granules. Therefore, the transformants were grouped into five classes based on the size of the blue-staining core (see Fig. 2), in which class I represents completely red-staining granules, and class V completely blue-staining ones (full phenotypic complementation). Figure 2 summarizes the performance of the different constructs in amylose synthesis in an *amf* background. A total of 340 transformants was analysed, about 80 per construct. Approximately 15% of the transformants showed no complementation at all (class I), whereas only 4% showed full phenotypic complementation (class V). The latter were only obtained after transformation with construct p↓PC protein. Clearly, the hybrid PC protein complemented to a higher extent than the unaltered CC protein. The use of either the cassava or the potato transit peptide did not show much difference in complementation efficiency; c↓CC seemed to perform slightly better than p↓CC. However, this efficiency was severely reduced with the tandem transit peptide (p↓c↓CC). As a reference, the complementation efficiency of the complete potato GBSS I (indicated by p↓PP) is also shown in Fig. 2 (based on Flipse *et al.* 1996b). It is apparent that in this case a much higher degree of complementation is achieved.

Further characterization of transformants

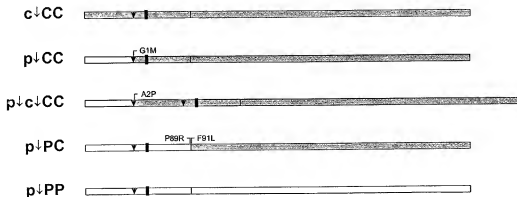From about 45 randomly selected transgenic potato plants, belonging to various complementation classes and

Figure 1. Schematic illustration of the proteins under investigation with their corresponding codes. Open boxes illustrate protein parts derived from stGBSS I, grey boxes those from meGBSS I. Arrow heads denote the splice site of the transit peptide.Black boxes represent the KTGGL motif which is the putative ADP-Glc binding site (Furukawa *et al.* 1990). Proteins are drawn to scale. Mutations as a result of sequence swapping are indicated.
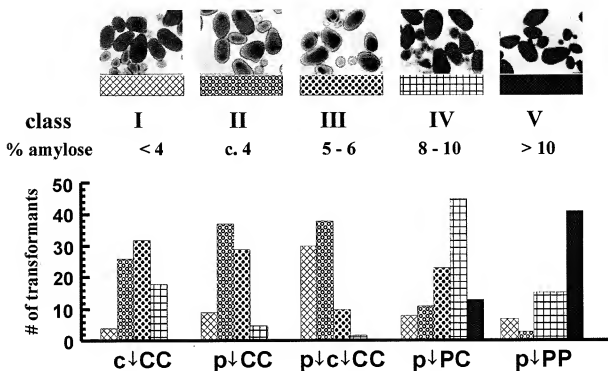
Figure 2. Overview of the number of transformants in the different complementation classes for c↓CC, p↓CC, p↓c↓CC, and p↓PC. As a reference, the performance of the complete potato GBSS I (p↓PP) has been extrapolated from a previous study (Flipse et al. 1996b). Note that in this case, class III and IV are shown as one bar. The assignment of classes from I (no complementation) to V (full phenotypic complementation) is indicated, together with an estimation of the corresponding amylose contents.

originating from transformations with constructs p↓CC, p↓c↓CC and p↓PC, the minimum number of T-DNA inserts was determined. This analysis showed that at least 1–6 T-DNA inserts were integrated in the different transformants. No relationship between the number of inserts and the degree of phenotypic complementation was found (results not shown).

Total RNA from the tubers of both the transgenic and the control potato plants was analysed by Northern blot analysis. Hybridization with the meGBSS I cDNA showed that similar amounts of the cassava transcript were produced in all transgenic plants, irrespective of their level of complementation (Fig. 3A). The only exceptions among the transformants tested were the class I p↓PC plants, which had a reduced level of the transcript. The cassava cDNA probe did not hybridize with the total RNA of the amf, wild-type and stGBSS I-complemented amf potato plants (further referred to as BW). This confirms that the cassava probe does not cross-react with the stGBSS I mRNA, which is present in all potato plants (also in the mutant background). Figure 3B showed that the stGBSS I transcript is present in all potato plants tested. Presumably, the stGBSS I probe cross-reacts with the meGBSS I mRNA, because the bands in the first three lanes of panel B stain at a much lower intensity than those in the other six, whereas the intensity of the bands in the last six lanes of panels A and B does not show much difference.

Granule-bound protein was extracted from the different classes of p↓PC transformants, as well as from the highest complementation class of the other transformants. Immunoblotting using antibodies raised against stGBSS I showed that the GBSS I protein (60 kDa) is present in class III to V p↓PC transformants (Fig. 4), but also in class IV c↓CC and class IV p↓CC (data not shown). The level of GBSSI protein in these transformants was slightly lower than those in the wild-type or BW starch granules, as can be judged from the intensity of the bands. No GBSS I protein was detected in the p↓c↓CC transformants, as well as (as expected) in the amf mutant. As can be seen from Fig. 4, the antibodies recognized a second granule-bound protein under the experimental conditions used. Considering its size, this protein is probably similar to SS II (sometimes refered to as GBSS II), which is very homologous to GBSS I except for its extra N-terminal extension, and which is known to be partially granule-bound (Edwards et al. 1995). It is obvious that the amount of SS II is not altered due to the transformation, and thus forms a reliable internal control for protein amounts applied to the gel.

The levels of amylose and GBSS I activity in transgenic potato plants

Amylose content and GBSS activity in the starch granules of the best performing transformants with c↓CC, p↓CC,
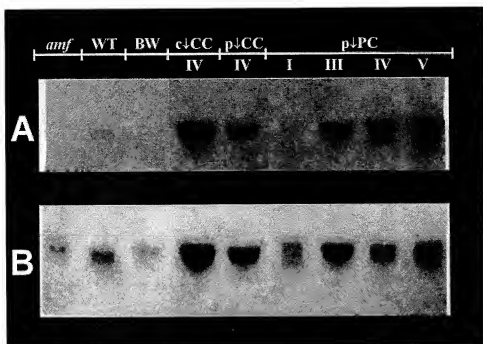
Figure 3. Northern blot analysis of tuber RNA extracted from controls (*amf*, wild-type and BW) and various individual transgenics carrying the indicated constructs and belonging to the different complementation classes. *amf*, amylose-free potato mutant; WT, wild-type potato; BW, *amf* mutant with full phenotypic complementation by potato GBSS I gDNA; The top panel was probed with meGBSS I cDNA; the bottom panel with potato GBSS I cDNA.
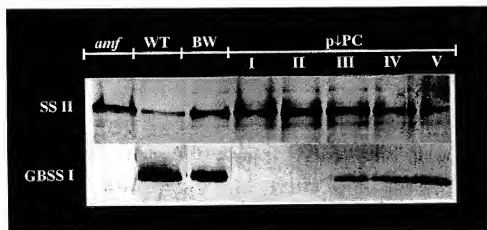
Figure 4. Western blot analysis of protein extracted from various starch granules. *amf*, amylose-free potato mutant; WT, wild-type potato; BW, *amf* mutant with full phenotypic complementation by potato GBSS I gDNA; I–V, p↓PC transgenics belonging to complementation classes I to V. Antibodies were raised against the GBSS I protein from potato.

and p↓c↓CC, as well as all transformants with p↓PC, are shown in Table 1. The amylose content was in agreement with the appearance of the granules upon iodine staining. From previous work (Kuipers *et al.* 1994), it is known that full phenotypic complementation corresponds to an amylose content greater than 10%. The best-performing plants (class V p↓PC) had 13% amylose compared to 22% in wild-type potato or the BW control plants. This represents a 60% restoration of amylose synthesis when com-

pared to the wild-type potato. This observation is remarkable because the GBSS I activity of these transformants is similar to or higher than in plants that have a normal amylose content (see also Fig. 5; note that the granules of BW plants have a higher GBSS I activity than those from wild-type plants).

In Fig. 5, the amylose content of the various starch granules is plotted against their GBSS I activity. A straight line can be fitted through these data points with a correlation

Table 1. Amylose content and GBSS I activity of starch granules from different transformants

Construct	Complementation class[b]	Amylose content[c]	% of BW	GBSS I activity[d]	% of BW
c↓CC	IV	8·2	37	46·8	54
p↓CC	IV	9·2	42	38·0	44
p↓c↓CC	IV	9·3	43	24·6	28
p↓PC	I	3·5	16	0·3	0
p↓PC	II	4·7	22	14·5	17
p↓PC	III	5·5	25	23·8	27
p↓PC	IV	8·2	38	53·6	62
p↓PC	V	13·0	60	56·0	64
amf	I	3·4	16	2·7	3
BW[a]	V	21·8	100	86·9	100

[a]BW is the *amf* control transformed with the genomic GBSS I gene from potato (Flipse *et al.* 1994). [b]For classification, see Fig. 2. Of each construct, transformants in the highest class were taken, except for p↓PC where examples of all classes were used. [c]Starch from tubers of a number of individual transformants belonging to this complementation class was pooled and assayed. The value is the mean of five individual determinations. [d]Starch from tubers of a number of individual transformants belonging to this complementation class was pooled and assayed. The value is the mean of three individual determinations. The activity is given as pmol Glc min^{-1} mg^{-1} starch.
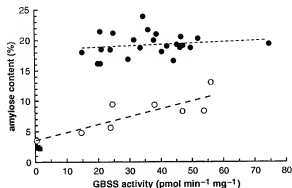
Figure 5. Relationship between the GBSS activity of several (transgenic) potato lines and their amylose content. (O) potato plants transformed with meGBSS I; (●), wild-type potato plants containing from 1–4 GBSS I alleles (derived from Flipse *et al.* 1996a); (■), amylose-free potato mutant.

coefficient of 0·936. This suggested that the amount of amylose produced is directly proportional to the GBSS I activity in the granules. Interestingly, such a linear correlation is not found in wild-type potato plants with a GBSS I activity ranging from 15 to 75 pmol min^{-1} mg^{-1} (Flipse *et al.* 1996a).

Physico-chemical characterization of the class V p↓PC starches

The physico-chemical characteristics of the class V p↓PC transgenic starches were mapped in some detail. Analysis

of the transgenic starch using a Bohlin rheometer showed a gradual increase in the storage modulus (G') upon cooling of the starch system. This increase was more prominent in wild-type starch and absent in the *amf* starch; it reflects retrogradation of amylose in the starch system. Differential scanning calorimetry showed an approximately 2 °C lower melting temperature (T_{onset}) of the class V p↓PC starch granules compared to that of the untransformed *amf* control (the T_{onset} of wild-type and *amf* potato starch is, respectively, 59·0 and 64·5 °C). The presence of amylose in starch is generally associated with a less organized (or less crystalline) structure of the granule, and consequently a lower T_{onset}.

The chain-length distribution of class V p↓PC, wild-type and *amf* starch was determined by HPAEC, after these starches had been debranched by isoamylase. No major differences in the HPAEC profiles were observed. This suggests that no large differences in the amylopectin fine structure are to be expected.

DISCUSSION

The present paper shows that the *amf* potato plant can be used as a heterologous host for the expression of foreign GBSS I genes. Depending on the construct used, the blue phenotype (but not the wild-type amylose content) of the granules could be restored in (at most) 13% of the transformants. In sharp contrast with this is the much higher percentage (62% instead of 13%) with full phenotypic complementation (and the wild-type amylose content) in *amf* plants transformed with stGBSS I gDNA (Flipse *et al.* 1996b). Apart from the deposition of amylose, no indications for an altered granule structure were found; the chain-

length distribution of the transformed, wild-type and *amf* starches was similar. Rheological and calorimetric analysis of the transgenic starches showed that their physico-chemical properties hold an intermediate position between wild-type and *amf* potato starch (Visser *et al.* 1997a,b), and are related to the amount of amylose. Two intriguing observations have been made during this work: (i) wild-type levels of amylose cannot be obtained by introducing a cassava GBSS I in potato, and (ii) the phenotype of the starch granules within one transformant is very consistent.

Southern analysis showed that at least 1–6 T-DNAs were inserted in the different transgenic potatoes. However, insertion of a meGBSS I gene did not always lead to amylose production; neither was there a relationship between the number of inserts and the level of complementation. Similar effects were observed in experiments where the stGBSS gDNA was expressed in its homologous (*amf* potato) host (van der Leij *et al.* 1991b; Flipse *et al.* 1994, 1996b). In addition to this, similar amounts of meGBSS I mRNA were found in most transgenics tested, thus there seems to be no relationship between the level of transcription and the amylose content. From this it is concluded that the incomplete restoration of amylose biosynthesis cannot be explained by the genetic constitution of the transgenic plants.

Although the potato and cassava GBSS I transit peptides have only 37% identical amino acids (Salehuzzaman *et al.* 1993), the latter is also suited for targeting meGBSS I to the potato amyloplast. In fact, the c↓CC construct seemed to perform slightly better than the p↓CC construct. Possibly this is due to a modification of the splice site in the p↓CC construct (as a result of introducing a *Sph*I site), where the original splice site motif IIC↓G was altered to IIC↓M. Doubling the length of the transit peptide of GBSS I (as in p↓c↓CC) apparently has a negative effect on amylose production; however, a small number of class IV transformants was obtained. It is unknown whether a two-step translocation is employed for p↓c↓CC during which the protein gets stuck in the membrane, thereby slowing down the meGBSS I influx into the amyloplast. Although the amount of GBSS I protein in the granules of class V transgenics is somewhat lower than that of the BW control granules, it seems unlikely that the incomplete restoration of the amylose content in the transgenics can be explained by non-functional transit peptides, especially when considering that GBSS activity is restored to normal values.

Analysis of *amf* gene dosage plants and stGBSS I-complemented *amf* potato plants has shown that large variations in GBSS activity (15–120 pmol Glc min⁻¹ mg⁻¹ starch) are tolerated in order to obtain wild-type levels of amylose (Flipse *et al.* 1994, 1996a). The GBSS activity of class IV and V transformants (25–55 pmol Glc min⁻¹ mg⁻¹ starch) falls well within this range (see Fig. 5). Thus, why is a surplus of GBSS activity unable to restore a wild-type level of amylose in the transgenic potato starch granules? This can only be explained by assuming that GBSS I from cassava and potato have different intrinsic properties. Although the amino acid sequences of the meGBSS I and

stGBSS I mature peptides are 80% similar, their charge at a given pH can differ considerably (pI values are 6·5 and 5·8, respectively). Consequently, both enzymes might for instance have different pH optima. Therefore, it could very well be that the meGBSS I operates at suboptimal conditions in the potato environment. In such a situation the amylose content may be proportional to the GBSS activity. In the case of stGBSS I in its own setting, the donor substrate ADP-Glc may be quickly depleted, and limit amylose synthesis. Here, a linear relationship between GBSS activity and amylose content is expected at much lower stGBSS I amounts. Analysis of antisense stGBSS I potato starch granules shows that this is indeed true (Kuipers *et al.* 1994).

Further evidence for different intrinsic properties of stGBSS I and meGBSS I is provided by our observation that the construct p↓PC performed better in complementation of *amf* starch than p↓CC or c↓CC. The hybrid mature protein PC contained the first 89 amino acids from stGBSS I, including the putative ADP-Glc binding site (Furukawa *et al.* 1990). It cannot be ruled out that the two amino acid modification in a highly conserved region of the GBSS I protein negatively affects the activity of PC. However, no large effect is expected since PC performs better than CC. Thus, engineering meGBSS I into a partial look-alike of stGBSS I seems to adapt this protein to the potato environment to a certain extent.

At first look, the phenotype of the starch granules of the meGBSS I-complemented *amf* plants seems very similar to that of wild-type potato plants in which the stGBSS I gene is antisense. However, an important difference is that the blue granule cores within each transformant of the former have approximately the same size, whereas this is not the case within each transformant of the latter. Apparently, antisense inhibition is a less reproducible event within one transformant than targeting a heterologous GBSS I protein to the potato amyloplast. Introduction of a homologous GBSS I gene in the *amf* potato mutant gave a much larger number of transformants showing a full complementation effect. However, in this case plants showing co-suppression also occur (Flipse *et al.* 1996b), yielding granules with a more variable core size than those transformed with the (heterologous) meGBSS I. In addition, co-suppression seems to depend on the conditions under which the plants were grown (Flipse *et al.* 1994). Thus, when engineering low-amylose (for instance 8%) potato starches is the objective, this is preferably done by introducing a heterologous GBSS I gene in an *amf* background. In this way, granules with an uniform amylose content are obtained which may be an advantage for processing or applications.

determinations, and T. van de Brink and J. Rijksen for taking care of the plants in the greenhouse.

REFERENCES

Bevan M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12,** 8711–8721.

Dellaporta S.L., Wood J. & Hicks J.B. (1983) A plant DNA minipreparation, version II. *Plant Molecular Biology Reports* **1,** 19–21.

Denyer K., Clarke B., Hylton C., Tatge H. & Smith A.M. (1996) The elongation of amylose and amylopectin chains in isolated starch granules. *Plant Journal* **10,** 1135–1143.

Edwards E., Marshall J., Sidebottom C., Visser R.G.F., Smith A.M. & Martin C. (1995) Biochemical and molecular characterization of a novel starch synthase from potato tubers. *Plant Journal* **8,** 283–294.

Flipse E., Huisman J.G., de Vries B.J., Bergervoet J.E.M., Jacobsen E. & Visser R.G.F. (1994) Expression of a wild-type GBSS gene introduced into an amylose-free potato mutant by *Agrobacterium tumefaciens* and the inheritance of the inserts at the microscopic level. *Theoretical and Applied Genetics* **88,** 369–375.

Flipse E., Keetels C.J.A.M., Jacobsen E. & Visser R.G.F. (1996a) The dosage effect of the wildtype GBSS allele is linear for GBSS activity but not for amylose content: absence of amylose has a distinct influence on the physico-chemical properties of starch. *Theoretical and Applied Genetics* **92,** 121–127.

Flipse E., Straatman-Engelen I., Kuipers A.G.J., Jacobsen E. & Visser R.G.F. (1996b) GBSS T-DNA inserts giving partial complementation of the *amylose-free* potato mutant can also cause co-suppression of the endogenous GBSS gene in a wild-type background. *Plant Molecular Biology* **31,** 731–739.

Furukawa K., Tagaya M., Preiss J. & Fukui Y. (1990) Identification of lysine 15 at the active site in *E. coli* glycogen synthase. *Journal of Biological Chemistry* **65,** 2086–2090.

Höfgen R. & Willmitzer L. (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Research* **16,** 9877.

Holmes D.S. & Quigley M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114,** 193–201.

Hovenkamp-Hermelink J.H.M., Jacobsen E., Ponstein A.S., Visser R.G.F., Vos-Scheperkeuter G.H., Bijmolt E.W., de Vries J.N., Witholt B. & Feenstra W.J. (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). *Theoretical and Applied Genetics* **75,** 217–221.

Hovenkamp-Hermelink J.H.M., de Vries J.N., Adamse P., Jacobsen E., Witholt B. & Feenstra W.J. (1988) Rapid estimation of the amylose/amylopectin ratio in small amount of tuber and leaf tissue of the potato. *Potato Research* **31,** 241–246.

Jacobsen E., Hovenkamp-Hermelink J.H.M., Krijgsheld H.T., Nijdam H., Pijnacker L.P., Wilthott B. & Feenstra W.J. (1989) Phenotypic and genotypic characterization of an amylose-free starch mutant of the potato. *Euphytica* **44,** 43–48.

Kortstee A.J., Vermeesch A.M.S., de Vries B.J., Jacobsen E. &

Visser R.G.F. (1996) Expression of *Escherichia coli* branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin. *Plant Journal* **10,** 83–90.

Kuipers A.G.J., Jacobsen E. & Visser R.G.F. (1994) Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. *Plant Cell* **6,** 43–52.

van der Leij F.R., Visser R.G.F., Ponstein A.S., Jacobsen E. & Feenstra W.J. (1991a) Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. *Molecular and General Genetics* **228,** 240–248.

van der Leij F.R., Visser R.G.F., Oosterhaven K., van der Kop D.A.M., Jacobsen E. & Feenstra W.J. (1991b) Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum* L.) by the gene encoding granule-bound starch synthase. *Theoretical and Applied Genetics* **82,** 289–295.

Murashige T. & Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Salehuzzaman S.N.I.M., Jacobsen E. & Visser R.G.F. (1993) Isolation and characterization of a cDNA encoding granule-bound starch synthase in cassava (*Manihot esculenta* Crantz) and its antisense expression in potato. *Plant Molecular Biology* **23,** 947–962.

Salehuzzaman S.N.I.M., Jacobsen E. & Visser R.G.F. (1994) Expression patterns of two starch biosynthetic genes in *in vitro* cultured cassava plants and their induction by sugars. *Plant Science* **98,** 53–62.

Smith A.M., Denyer K. & Martin C. (1997) The synthesis of the starch granule. *Annual Reviews in Plant Physiology and Plant Molecular Biology* **48,** 67–87.

Visser R.G.F. (1991) Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual,* **B5** (ed. K. Lindsey), pp. 1–9. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Visser R.G.F., Stolte A. & Jacobsen E. (1991) Expression of a chimaeric granule-bound starch synthase-GUS gene in transgenic potato plants. *Plant Molecular Biology* **17,** 691–699.

Visser R.G.F., Suurs L.C.J.M., Bruinenberg P.M., Bleeker I. & Jacobsen E. (1997a) Comparison between amylose-free and amylose containing potato starches. *Starch* **49,** 438–443.

Visser R.G.F., Suurs L.C.J.M., Steeneken P.A.M. & Jacobsen E. (1997b) Some physicochemical properties of amylose-free potato starch. *Starch* **49,** 443–448.

Vos-Scheperkeuter G.H., de Boer W., Visser R.G.F., Feenstra W.J. & Witholt B. (1986) Identification of granule-bound starch synthase in potato tubers. *Plant Physiology* **82,** 411–416.

van de Wal M., D'Hulst C., Vincken J.-P., Buléon A., Visser R.G.F. & Ball S. (1998) Amylose is synthesized *in vitro* by extension of and cleavage from amylopectin. *Journal of Biological Chemistry* **273,** 22232–22240.

(10)   Related Proceedings Appendix

None.